

University of Southern Queensland  
Faculty of Engineering and Surveying

**HOW EFFECTIVE IS MICROALGAE  
TREATMENT OF DIFFERENT WASTEWATERS  
FOR SIMULTANEOUS NUTRIENT REMOVAL  
AND LIPID PRODUCTION FOR BIO-FUEL?**

A dissertation submitted by

Nadine Pufelski

in fulfillment of the requirements of

**Courses ENG4111 and 4112 Research Project**

towards the degree of

**Bachelor of Engineering (Environmental)**

Submitted: October, 2010

# ABSTRACT

Nutrient removal from wastewaters has been traditionally achieved by activated sludge. In recent years, research has focused on the use of microalgae to simultaneously achieve nutrient removal from wastewaters and lipid production for use as bio-fuel. The effluent that has been polished by algae treatment can be recycled for different beneficial uses. If carbon taxes are introduced, bio-diesel production through algae can potentially become lucrative as this process is virtually carbon neutral when used in conjunction with CO<sub>2</sub> mitigation. There are studies available on algae production using piggery, dairy and municipal wastewater but the optimisation of lipid production is yet to be carried out. In addition, the growth rates of algae in different wastewaters and potential lipid production have not been fully established.

Hence, in this dissertation, the aim was to investigate the rates of nutrient removal, algal growth and lipid production using the wastewater collected from a leafy vegetable nursery, a wastewater reclamation facility and a dairy. Experiments were conducted using a batch reactor, having a capacity of 3.5 L. The reactor was filled with the wastewater and inoculated with *Chlorella vulgaris* microalgae. Both compressed air and CO<sub>2</sub> were supplied to provide the carbon necessary for the algal growth. The required illumination for growth was provided by florescent light. Both pH and DO were monitored every minute and the pH was controlled at a set-point of  $7.5 \pm 0.5$ . Data acquisition of the analogue signals from the pH and DO sensors was processed by a personal computer equipped with Labview software. The algal growth rate was monitored by measuring suspended solid concentration and optical density using a spectrophotometer. When the growth entered the death phase, the algae were harvested for lipid measurement.

Experimental results indicated that algae can indeed remove nitrogen from nursery, municipal and dairy wastewater at rates of 2.64 mg/L/d, 1.59 mg/L/d and 1.73 mg/L/d respectively. However, nitrification was also detected in all three wastewaters. Phosphorous removal rates from nursery and municipal wastewater were 0.27 mg/L/d and 0.51 mg/L/d which resulted in a N:P utilisation ratio of 49:5 and 3:1 respectively. Lipid production was

found to be most successful in nursery wastewater with a maximum total lipid content of 25.5 % of the algal dry weight, followed by municipal wastewater with a maximum of 12.8 % total lipids of the algal dry weight. The total lipid content benchmark was 20-30 % for *Chlorella vulgaris*. These results obtained from the batch experiments are very promising whereby *Chlorella vulgaris* microalgae can be successfully utilised for nutrient removal and lipid production from different wastewater.

This research will give insight into the feasibilities of using small scale effluent cleaning via algal growth in decentralised businesses, which have the potential to have their own on-site algae farm for producing bio-fuels and CO<sub>2</sub> mitigation.

University of Southern Queensland  
Faculty of Engineering and Surveying

<b>ENG4111 and 4112 Research Project</b>
--

### **Limitations of Use**

The Council of the University of Southern Queensland, its Faculty of Engineering and Surveying, and the staff of the University of Southern Queensland, do not accept any responsibility for the truth, accuracy or completeness of material contained within or associated with this dissertation.

Persons using all or any part of this material do so at their own risk, and not at the risk of the Council of the University of Southern Queensland, its Faculty of Engineering and Surveying, and the staff of the University of Southern Queensland.

This dissertation reports an educational exercise and has no purpose or validity beyond this exercise. The sole purpose of the course pair entitled “Research Project” is to contribute to the overall education within the student’s chosen degree program. This document, the associated hardware, software, drawings, and other material set out in the associated appendices should not be used for any other purpose: if they are so used, it is entirely at the risk of the user.



**Prof Frank Bullen**

Dean

Faculty of Engineering and Surveying

# **CERTIFICATION**

I certify that the ideas, designs and experimental work, results, analyses and conclusions set out in this dissertation are entirely my own effort, except where otherwise indicated and acknowledged.

I further certify that the work is original and has not been previously submitted for assessment in any other course or institution, except where specifically stated.

**Nadine Pufelski**

**Student Number: 0050062698**

---

Signature

---

Date

# ACKNOWLEDGEMENT

I would like to thank Dr Vasanthadevi Aravinthan for her dedicated supervision as well as valuable and passionate guidance provided during the undertaking of this project. Without her help and encouragement this project would not have been as successful and fun. I would also like to thank Dr Talal Yusaf who so kindly provided me with an additional supervisor and mentor.

Special thanks go to Peter Harris who provided substantial guidance, generous time and a helping hand with the biology and chemistry laboratory equipment and techniques throughout this project.

I wish to extend my sincere gratitude to Nishant Pradhan who so patiently assisted me all through the project with setting up the reactor and the ion-analyser for my large amount of samples.

I also want to thank Saddam Hussen Allwayzy, Raed Ahmed Mahmood, Nick Stuckey, Muhammad Azizul Hoque, Dean Beliveau, Adrian Blokland, Adele Jones, Morwenna Boddington, Michael Hobson, Kim Larsen and Geoff O'Shannassy for their contribution and assistance. To the Toowoomba Regional Council, the Story family and the Kunde family, I wish to extend my sincere appreciation for supplying me with wastewater.

My final thanks go to my family, friends, Matilda and Brent Story for their continuous support throughout this project and my time at university.

# GLOSSARY

The following abbreviations are used throughout the dissertation:

<b>TN</b>	Total nitrogen
<b>IC</b>	Ion chromatography
<b>DO</b>	Dissolved oxygen
<b>TOC</b>	Total organic carbon
<b>BOD</b>	Biochemical oxygen demand
<b>COD</b>	Chemical oxygen demand

# CONTENTS

<b>Abstract</b>	i
<b>Certification</b>	iv
<b>Acknowledgement</b>	v
<b>Glossary</b>	vi
<b>List of Figures</b>	xii
<b>List of Tables</b>	xvii
<b>Chapter 1 Introduction and Goals</b>	1
1.1 Aims and Objectives	2
1.2 Scope of Study	3
1.3 Dissertation Outline	3
<b>Chapter 2 Literature Review</b>	5
2.1 Microalgae as a Fuel Source	5
2.1.1 <i>Review of Algal Lipid Production and Nutrient Uptake Research</i>	7
2.2 Additional Uses of Microalgae By-Products	10
2.3 Microalgae as a Vehicle for Decentralisation	10
2.4 <i>Chlorella vulgaris</i> as a Typical Algal Strain Used for culturing	11
2.5 Typical Culturing Techniques	12
2.5.1 <i>Nutrient Requirements and Ratios</i>	12
2.5.2 <i>Carbon Dioxide and pH Requirements</i>	13
2.5.3 <i>Light Requirements</i>	14
2.5.4 <i>Temperature Requirements</i>	15
2.5.5 <i>Organic Load Tolerances</i>	15
2.5.6 <i>Mixing Techniques</i>	16
2.5.7 <i>Algae Recovery and Harvesting</i>	16
2.6 Microalgae Growth Phases	17



2.7	Photosynthesis in Microalgae .....	18
2.8	Chapter Summary.....	21
<b>Chapter 3</b>	<b>Methodology .....</b>	<b>22</b>
3.1	Wastewater Collection and Preparation .....	22
3.1.1	Nursery Runoff .....	22
3.1.2	Lettuce Factory Wastewater .....	23
3.1.3	Municipal Wastewater .....	23
3.1.4	Dairy Wastewater.....	23
3.1.5	Preparation and Storage .....	23
3.2	Wastewater Characteristics .....	24
3.2.1	Special adjustments to the wastewater media.....	26
3.3	Bio-Reactor Design .....	28
3.4	Pre-Culturing and Inoculation of <i>Chlorella vulgaris</i> .....	31
3.4.1	Preparation of MBL .....	31
3.4.2	Pre-Culturing of <i>Chlorella vulgaris</i> .....	32
3.4.3	Inoculation of <i>Chlorella vulgaris</i> into the Bio-Reactor.....	32
3.5	Experimental measurements .....	33
3.5.1	Measurement of Algal Growth .....	33
3.5.2	Measurement of Nutrient Depletion .....	34
3.5.3	Measurement of Online DO and pH Data.....	34
3.5.4	Microalgae Harvest.....	36
3.5.5	Determination of Algal Dry Weight .....	37
3.5.6	Determination of Total Lipid Content .....	37
3.5.7	Polar and Neutral Lipids Separation .....	38
3.5.8	Transesterification.....	39
3.6	Data Analysis .....	40
3.6.1	Nutrient Utilisation Calculations .....	40

3.6.2	Specific Growth Rate Calculations .....	41
3.6.3	Online Data Manipulation.....	42
3.7	Risk Minimisation .....	43
3.7.1	Clogging of Drains due to Algae Growth .....	43
3.7.2	Bacterial Management .....	43
3.7.3	Disease Prevention.....	43
3.8	Chapter Summary.....	43
<b>Chapter 4 Nutrient Removal and Lipid Production of <i>Chlorella vulgaris</i> Microalgae Grown in Nursery Runoff Wastewater .....</b>		<b>44</b>
4.1	Batch Experiment 1 (Preliminary experiment) .....	44
4.2	Batch Experiment 2 - Raw Wastewater Characteristics.....	46
4.3	Growth Characteristics .....	47
4.4	Nutrient Removal .....	50
4.5	pH Change with Time .....	52
4.6	Dissolved Oxygen Change with Time .....	54
4.7	Algal Dry Mass and Lipid Content .....	57
4.8	Limiting Factors of Algal Growth.....	58
4.9	Summary .....	59
<b>Chapter 5 Nutrient Removal and Lipid Production of <i>Chlorella vulgaris</i> Microalgae Grown in Municipal Wastewater .....</b>		<b>60</b>
5.1	Batch Experiment 1 (Preliminary experiment) .....	60
5.2	Batch Experiment 2 - Raw Wastewater Characteristics.....	61
5.3	Growth Characteristics .....	63
5.4	Nutrient Removal .....	66
5.5	pH Change with Time .....	69
5.6	Dissolved Oxygen Change with Time .....	72
5.7	Algal Dry Mass and Lipid Content .....	75
5.8	Limiting Factors of Algal Growth.....	75

5.9	Summary .....	77
<b>Chapter 6 Nutrient Removal and Lipid Production of <i>Chlorella vulgaris</i> Microalgae Grown in Dairy Wastewater .....</b>		
		78
6.1	Batch Experiment 1 (Preliminary experiment) .....	78
6.2	Batch Experiment 2 - Raw Wastewater Characteristics.....	79
4.3	Growth Characteristics .....	81
6.4	Nutrient Removal .....	84
6.5	pH Change with Time .....	86
6.6	Dissolved Oxygen Change with Time .....	88
6.7	Algal Dry Mass and Lipid Content .....	90
6.8	Limiting Factors of Algal Growth.....	91
6.9	Summary .....	92
<b>Chapter 7 Comparative Study Between Different Wastewaters .....</b>		
		93
7.1	Comparison of Growth.....	93
7.2	Comparison of Nutrient Utilisation.....	95
7.2.1	Utilisation of Nitrogen .....	95
7.2.2	Utilisation of Phosphorous.....	96
7.3	Comparison of CO <sub>2</sub> Uptake Abilities.....	97
7.4	Comparison of Lipid Production.....	98
7.5	Summary .....	102
<b>Chapter 8 Conclusions and Future Work.....</b>		
		103
8.1	Conclusions .....	103
8.2	Suggestions for Future Work .....	105
8.2.1	Controls and Additional Experiments.....	105
8.2.2	Improved Carbon Mitigation and pH Control .....	105
8.2.3	Reduction of Bacterial Competition .....	106
8.2.4	Improved Reactor Design .....	106

8.2.5	Improved Identification of Algal Growth .....	106
8.3	Summary .....	107
<b>References</b>	.....	108

<b>Appendix A</b>	<b>Project Specifications</b>
<b>Appendix B</b>	<b>Growth and Nutrient Depletion Data</b>
<b>Appendix C</b>	<b>Sample Matlab Code</b>

# LIST OF FIGURES

Figure 2.1	<i>Chlorella</i> (Source: A beginner's guide to Freshwater Algae 1987).....	11
Figure 2.2	Growth phases of microalgae .....	18
Figure 2.3	Conversion of light energy to chemical energy in the light reaction (adopted from Forbes & Watson 1992).....	19
Figure 3.1	Total Organic Carbon/ Total Nitrogen Analyzer (TOC-VCPH/CPN) testing a sample.....	24
Figure 3.2	Sample calculation for preparing the wastewater media.....	25
Figure 3.3	Schematic diagram of the bio-reactor .....	29
Figure 3.4	Experimental set-up.....	30
Figure 3.5	Young and older culture of <i>Chlorella vulgaris</i> in MBL in Erlenmeyer flasks with cotton buds .....	32
Figure 3.6	Data output by the Jenway 6705 UV/Vis. Spectrophotometer.....	33
Figure 3.7	5ml samples for Ion Chromatography system (IC, Dionex ICS 2000) measurements (left) and ≈10ml samples for Nitrogen Analyzer (TOC-VCPH/CPN) measurements (right).....	34
Figure 3.8	The Labview software package logging DO, pH, acid and base addition and time.....	35
Figure 3.9	Freeze dryer (VirTis 2KBTES-55).....	36
Figure 3.10	A nitrogen stream was used to dry the lipids.....	38
Figure 3.11	Column chromatography was performed to separate neutral and polar lipid.....	39
Figure 3.12	The neutral lipid-chloroform mix is distilled in a Florence flask to speed up the drying process of the lipids.....	39
Figure 4.1	Oxygen bubbles on the DO probe.....	45

Figure 4.2	Algal growth in nursery wastewater was monitored by observing daily changes by measuring the absorbance at the optical density at 505 nm with a spectrophotometer (Jenway 6705 UV/Vis.) and suspended solids.....	47
Figure 4.3	Comparison of algal growth between the first and second nursery batch experiment. Growth was measured with optical density at 505 nm with a spectrophotometer (Jenway 6705 UV/Vis.) and suspended solids .....	48
Figure 4.4	Relationship between suspended solids measurements and optical density at 505nm using nursery wastewater.....	49
Figure 4.5	Filter paper collected over the growth period in nursery wastewater, where the numbers represent the day of filtration.....	50
Figure 4.6	The graph shows the change in nitrogen concentration in form of Org-N + NH <sub>4</sub> -N, TN, NO <sub>2</sub> -N, NO <sub>3</sub> -N using nursery wastewater.....	51
Figure 4.7	PO <sub>4</sub> -P change with time using nursery wastewater.....	52
Figure 4.8	pH was monitored every minute for 18 days using nursery wastewater. ....	53
Figure 4.9	The graph shows the pH increase due to CO <sub>2</sub> consumption by the algae during the light period and the decrease of pH due to CO <sub>2</sub> addition for different growth phases: lag phase, linear phase, stationary phase and death phase using nursery wastewater. ....	54
Figure 4.10	Dissolved oxygen was adjusted to 20 degrees Celsius and monitored every minute for 18 days using nursery wastewater. The bottom graph indicates light and dark periods. ....	55
Figure 4.11	The graph shows the average maximum dissolved oxygen reading during the light period, the average minimum dissolved oxygen reading during the dark period, the net oxygen produced during the daytime and the optical density readings using nursery wastewater. ....	56
Figure 4.12	The graph shows the change in dissolved oxygen concentration in the bio-reactor for different growth phases: lag phase, linear phase, stationary phase and death phase using nursery wastewater. ....	57
Figure 4.13	Freeze dried algal mass in a petri dish.....	58

Figure 5.1	Algal growth in municipal wastewater was monitored by observing daily changes by measuring the absorbance at the optical density at 505 nm with a spectrophotometer (Jenway 6705 UV/Vis.) and suspended solids.....	64
Figure 5.2	<i>Chlorella vulgaris</i> in municipal wastewater with sedimentation on the reactor housing and on the floor. ....	64
Figure 5.3	Relationship between suspended solids measurements and optical density at 505nm using municipal wastewater. ....	65
Figure 5.4	Filter paper collected over the growth period, where the numbers represent the day of filtration using municipal wastewater. ....	66
Figure 5.5	The graph shows the change in nitrogen concentration in form of Org-N + NH <sub>4</sub> -N, TN, NO <sub>2</sub> -N, NO <sub>3</sub> -N using municipal wastewater. ....	67
Figure 5.6	Nitrite (NO <sub>2</sub> -N) change with time.....	68
Figure 5.7	PO <sub>4</sub> -P change with time using municipal wastewater. ....	68
Figure 5.8	pH was monitored every minute for 12 days using municipal wastewater. ....	69
Figure 5.9	The graph shows the pH increase due to CO <sub>2</sub> consumption by the algae during the light period and the decrease of pH due to CO <sub>2</sub> addition for different growth phases: linear phase, stationary phase and death phase using municipal wastewater. ....	71
Figure 5.10	Algal settlement on pH meter. ....	71
Figure 5.11	Dissolved oxygen was adjusted to 20 degrees Celsius and monitored every minute for 12 days. The bottom graph indicates light and dark periods.....	72
Figure 5.12	The graph shows the average maximum dissolved oxygen reading during the light period, the average minimum dissolved oxygen reading during the dark period, the net oxygen produced during the daytime and the optical density readings using municipal wastewater. ....	73
Figure 5.13	The graph shows the change in dissolved oxygen concentration in the bio-reactor for different growth phases: linear phase, stationary phase and death phase using municipal wastewater. ....	74
Figure 5.14	Bacteria colonies after 48 hours from a municipal sample one day 10 after inoculation Difco <sup>MT</sup> Nutrient Agar.....	76

Figure 5.15	<i>Chlorella vulgaris</i> in municipal wastewater (after the batch experiment concluded) under 1000 x magnification. In comparison healthy <i>Chlorella vulgaris</i> cells in MBL under 1000 x magnification (small picture). ....	76
Figure 6.1	2.5 x diluted dairy wastewater in bio-reactor.....	79
Figure 6.2	Algal growth in dairy wastewater was monitored by observing daily changes by measuring the absorbance at the optical density at 505 nm with a spectrophotometer (Jenway 6705 UV/Vis.) and suspended solids.....	82
Figure 6.3	Relationship between suspended solids measurements and optical density at 505nm using dairy wastewater.....	83
Figure 6.4	Filter paper collected over the growth period, where the numbers represent the day of filtration using dairy wastewater. ....	84
Figure 6.5	The graph shows the change in nitrogen concentration in form of Org-N + NH <sub>4</sub> -N, TN, NO <sub>2</sub> -N, NO <sub>3</sub> -N using dairy wastewater. ....	85
Figure 6.6	Change in Nitrite and Nitrate over the growth period.....	85
Figure 6.7	PO <sub>4</sub> -P change with time using dairy wastewater.....	86
Figure 6.8	pH was monitored every minute for 16 days using dairy wastewater. ....	87
Figure 6.9	The graph shows the pH increase due to CO <sub>2</sub> consumption by the algae during the light period and the decrease of pH due to CO <sub>2</sub> addition for different growth phases using dairy wastewater: lag phase, linear phase, stationary phase and death phase. ....	88
Figure 6.10	Dissolved oxygen was adjusted to 20 degrees Celsius and monitored every minute for 16 days. The bottom graph indicates light and dark periods using dairy wastewater. ....	89
Figure 6.11	The graph shows the change in dissolved oxygen concentration in the bio-reactor for different growth phases using dairy wastewater: lag phase, linear phase, stationary phase and death phase. ....	90
Figure 6.12	<i>Enterobacteriaceae</i> bacteria on CM0007 MacCONKEY agar after 24 hours from dairy wastewater on day 5 after inoculation.....	91
Figure 6.13	<i>Staphyrococcus</i> species on CM0007 MacCONKEY agar... ..	91
Figure 6.14	Algal growth on the reactor walls, observed after emptying of the reactor....	92



Figure 7.1	Comparison of growth behaviour in nursery, municipal and dairy wastewater in form of optical density and suspended solids.....	94
Figure 7.2	Comparison of total nitrogen removal rates .....	96
Figure 7.3	Comparison of Org. + NH <sub>4</sub> nitrogen removal rates .....	96
Figure 7.4	Comparison of nitrate nitrogen removal rates.....	96
Figure 7.5	Comparison of phosphate phosphorous removal rates.....	97
Figure 7.6	Comparison of CO <sub>2</sub> uptake abilities.....	98
Figure 7.7	Lipid percentages from this research and external research.....	101
Figure 7.8	Total lipid masses obtained from this research.....	101

# LIST OF TABLES

Table 2.1	Comparison of some bio-fuel sources (Chisti 2007).....	6
Table 2.2	Previously researched lipid content of <i>C. vulgaris</i> in different media.....	8
Table 2.3	Previously researched algae growth and nutrient removal in different wastewaters.....	10
Table 2.4	Doubling times and lipid contents of different algal strains.....	12
Table 3.1	Components for the preparation of MBL medium.....	31
Table 4.1	Wastewater characteristics obtained from the nursery runoff, fertilizer tank and lettuce factory.....	46
Table 4.2	Selected wastewater mix with final nutrient values and organic strength.....	46
Table 5.1	Wastewater characteristics obtained from the clarifier and influent.....	62
Table 5.2	Selected wastewater mix with final nutrient values and organic strengths.....	63
Table 6.1	Wastewater characteristics obtained from the dairy wastewater.....	80
Table 6.2	Selected wastewater mix with nutrient values and organic strengths.....	81

# CHAPTER 1 INTRODUCTION AND GOALS

In recent years, there has been a strong focus on the development environmental-friendly renewable sources of energy, driven by the need for sustainable fuel sources. A key focus in the development of new technology has been to identify “green” solutions which minimise greenhouse gases. Currently 80% of energy production utilises fossil fuels (Wiggers et al 2009), with new alternative energy sources seeking to reduce our reliance on fossil energy resources. Another important move towards sustainability has been to produce cleaner waste products across all industries. In the wastewater industry, the focus is on nutrient removal. One option to produce a sustainable fuel source and to concurrently achieve nutrient removal in waste streams is growing microalgae for bio-fuel production. The removal of nutrients in effluents is important to reduce the potential of an outbreak of undesirable algae that can make the water unfit for any beneficial use and that can also cause drastic reduction of dissolved oxygen in the water body. Nutrient removal from wastewaters has been traditionally achieved by activated sludge. In recent years, microalgae have been exploited for simultaneous nutrient removal from wastewaters that need polishing and lipid production for bio-fuel. Effluent polished by algae treatment can be recycled for different beneficial uses which may include irrigation water, stream water or toilet waste removal. If carbon taxes are introduced, bio-diesel production through algae can potentially become very lucrative as this process is virtually carbon neutral when used in conjunction with CO<sub>2</sub> removal (Li et al 2008).

Another important development has been the trend towards decentralisation. The concept can best be described by “live, work, produce, learn and shop in the same place” (Neef 2007). This can reduce transportation and energy usage, hence it is more sustainable and energy efficient. Ideally, the algae fuel production, carbon mitigation and wastewater polishing can also be decentralised where by different industries can have an onsite algae operation using their own wastewater for bio-fuel production.

There are limited studies available on algae production using piggery, dairy and municipal wastewater, but rates of nutrient removal and algal growth have not been fully investigated.

Furthermore, the growth rates of algae in different wastewaters for potential lipid production have not been fully established. Hence, this dissertation critically reviews the research previously conducted in the field of nutrient removal and lipid production through the use of microalgae on wastewaters. In addition, it is aimed to investigate the rates of nutrient removal and algal growth using the wastewater collected from a leafy vegetable nursery, a wastewater reclamation facility and a dairy farm. The research can give insight into the feasibilities of using small scale effluent cleaning via algal growth in decentralised businesses, which have the potential to have their own on-site algae farm for polishing effluent and producing algae for bio-fuel.

## 1.1 AIMS AND OBJECTIVES

The aim for this project was to determine whether a range of effluent producers are able to remove nutrients in their wastewaters, offset CO<sub>2</sub> and produce sufficient quantities and quality of bio-diesel feedstock. The three wastewaters that were chosen for this study are leafy vegetable nursery runoff, municipal wastewater and dairy wastewater.

The objectives of this research were to:

- Obtain wastewater samples from the above mentioned locations and test those samples for their nutrient and organic load characteristics
- Grow *Chlorella vulgaris* microalgae in the wastewater samples and simultaneously measure
  - algal growth
  - pH variations
  - dissolved oxygen variations
  - nutrient (nitrogen and phosphorous) variations (depletions);
- Identify growth patterns, nutrient depletion as well as pH and DO patterns; and
- Harvest the algae and measure total and neutral lipid production.

## 1.2 SCOPE OF STUDY

The scope of this research is to identify the suitability of undertaking nutrient removal, CO<sub>2</sub> mitigation and bio-diesel feedstock production using *Chlorella vulgaris* microalgae in a number of different wastewaters.

The limitations of this research were:

- Only three different wastewaters were chosen for this study (nursery, municipal and dairy)
- Each wastewater was tested only twice, where the initial test was used to identify pH, DO and algal growth behaviour
- No control media was used
- The amount of CO<sub>2</sub> feeding had to be adjusted for every wastewater due to pH limitations, resulting in different carbon feeding rates
- Much of the system was identified to represent a black box, where most of the input and output parameters were known, but the individual chemical and biological reactions could not be described.

## 1.3 DISSERTATION OUTLINE

The remainder of the dissertation is structured as follows:

### *Chapter 2 Literature Review*

This chapter reviews and summarises literature relating to the beneficial uses of microalgae, typical culturing techniques and algal growth phases. It also gives a brief overview of photosynthesis and the resulting DO production and pH fluctuations.

### *Chapter 3 Methodology*

This chapter gives an overview of the methodology used to analyse the wastewater characteristics, growing conditions, and nutrient depletions. It also shows the experimental set up and lipid analysis techniques.

*Chapter 4      Nutrient Removal and Lipid Production of Chlorella vulgaris Microalgae  
Grown in Nursery Runoff Wastewater*

Chapter 4 presents the results relating to the testing of nursery runoff wastewater. It shows growth patterns, pH variations, and changes in dissolved oxygen, nutrient depletions and lipid production. The chapter also discusses possible reasons for algal death after a number of days.

*Chapter 5      Nutrient Removal and Lipid Production of Chlorella vulgaris Microalgae  
Grown in Municipal Wastewater*

Chapter 5 presents the results relating to the testing of municipal wastewater. It shows growth patterns, pH variations, changes in dissolved oxygen, nutrient depletions and lipid production. The chapter also discusses possible reasons for algal death after a number of days.

*Chapter 6      Nutrient Removal and Lipid Production of Chlorella vulgaris Microalgae  
Grown in Dairy Wastewater*

Chapter 6 presents the results relating to the testing of dairy wastewater. It shows growth patterns, pH variations, changes in dissolved oxygen, nutrient depletions and lipid production. The chapter also discusses possible reasons for algal death after a number of days.

*Chapter 7      Comparative Study between Different Wastewaters*

This chapter compares and contrasts the results obtained from the three different wastewaters investigated above.

*Chapter 8      Conclusions and Future Work*

Finally the conclusions of this study and future work are presented in Chapter 8.

## CHAPTER 2 LITERATURE REVIEW

This literature review covers the uses of microalgae as fuel, wastewater polisher and CO<sub>2</sub> sink, the research carried out to date and a number of culturing techniques for microalgae. In addition, an overview of *Chlorella vulgaris* microalgae and the reactions involved in photosynthesis are provided.

### 2.1 MICROALGAE AS A FUEL SOURCE

Bio-fuels are in large demand as they appear to be lucrative and environmentally friendly. But the intensive use of bio-fuels made from terrestrial oil plants can also cause significant damage to the environment and the world's food supply. Fuel crops such as corn, sunflower or canola can possibly become a significant competitor with food crops for the use of arable land (Li 2009). This could drastically threaten the world's food supply. In drought affected countries such as Australia, growing feedstock for the fuel production can become very unreliable, and expensive grain imports may be necessary (Li 2009). Profitable oil crops can also pose a significant environmental risk in tropical countries, where rainforests are often cleared to make way for crop land.

The use of microalgae as bio-fuel may provide a solution to this problem. These organisms are one of the simplest and oldest of the world (Li 2009). They only require water, sunlight, carbon dioxide and a supply of nutrients such as nitrogen, phosphates and some trace minerals for example iron (Li 2009). Microalgae can be grown in wastewaters, coal seam gas waters, heavy metal waters and wastewater at elevated temperatures (Benemann & Oswald 1994) which means that no primary farmland is required (Park et al 2009). Hence, algae do not compete with food crops. Algae have a higher lipid content and grow much faster compared to the traditionally grown oil crops such as sunflower and oil palm (Chisti 2007). As a result, microalgae were identified as the only possible alternative fuel sources to one day replace the entire fossil diesel demand of the United States (Li et al 2008, Chisti 2007). According to Chisti (2007), microalgae can also perform exceptionally well in terms of oil yield. Where corn, canola or oil palms have an oil yield of 172 L/ha/yr, 110 L/ha/yr and 5950 L/ha/yr respectively, microalgae can produce up to 136 900 L/ha/yr depending on

species and conditions (here microalgae with 70% oil per dry weight). Table 2.1 shows a range of possible oil crops for the bio-fuel production. The land area needed refers to the area needed to meet 50% of all transport related fuel needs of the United States.

TABLE 2.1 COMPARISON OF SOME BIO-FUEL SOURCES (CHISTI 2007)

<b>Crop</b>	<b>Oil yield (L/ha)</b>	<b>Land area needed (million ha)</b>
Corn	172	1540
Soy bean	446	594
Canola	1190	223
Coconut	2689	99
Oil palm	5950	45
Microalgae with 70% oil per dry weight	136900	2
Microalgae with 30% oil per dry weight	58700	4.5

When burnt, bio-fuels from algae produce 70 % less greenhouse gas emissions compared to fossil fuels (SARDI 2009) and minimise the release of nitrous oxides, sulfur and other gaseous pollutants (Li et al 2008, Widjaja et al 2009, Sheehan et al 1998, Mata et al 2010).

Microalgae were first grown in the 1950's, when they were initially identified as a potential food source for humans and animals (Park et al 2009, Sheehan et al 1998). During the fuel crisis in the 1970's, researchers began to evaluate the potential for using algae in the production of bio-diesel (Li et al 2008, Sheehan et al 1998). Now microalgae are being reinvestigated as a fuel source, wastewater polisher and CO<sub>2</sub> sink. Microalgae research is now underway in at least 11 countries including Germany, USA, Japan, Australia, China, Taiwan, Turkey and India (Wellinger 2009).



### 2.1.1 REVIEW OF ALGAL LIPID PRODUCTION AND NUTRIENT UPTAKE RESEARCH

According to Johnson and Sprague (1987), algae produce and store lipids to survive when food supply reduces. During that time, they stop growing and dividing. More specifically, Widjaja et al (2009) suggested that the depletion of nitrogen induces the lipid accumulation. They found that 17 days of nitrogen depletion gave the best results for lipid levels in their experiments. They reported a drop in lipid levels after only 7 days depletion; however the lipid amount rose again after that time.

Woertz et al (2009) measured the lipid content in dairy and municipal wastewater. They trialed a number of hydraulic retention times and CO<sub>2</sub> or air feeding rates. Their lipid content in the municipal wastewater ranged from 29.2 to 73.3mg/L. The algal lipid content ranged from 4.9 % to 11.3 %, increasing with reducing hydraulic retention times. Table 2.2 shows several additional results for total lipids and neutral lipids in *Chlorella vulgaris* microalgae. De-Bashal et al (2002) only found 0.01 % total lipids in their algae. But the small amount of lipids may suggest that some data was presented incorrectly or that they cultured only very small amounts of algae, resulting in large experimental error. Rodolfi et al (2009) grew *Chlorella vulgaris* in a nutrient deplete medium and achieved 18 % total lipids. Widjaja et al (2009) even achieved 52 % in their modified Fitzgerald medium. Converti et al (2009) and Putt (2007) reported maximum values of 15 % and 30 % respectively. Chen et al (2009) achieved a neutral lipid content of 56 % and Harris (2010) reported 5 % total lipids and 2 % neutral lipids in MBL cultured *Chlorella vulgaris*.

TABLE 2.2 PREVIOUSLY RESEARCHED LIPID CONTENT OF *C. VULGARIS* IN DIFFERENT MEDIA

Algal strain	Growth medium	Total lipids as % dry weight of biomass	Neutral lipids as % dry weight of biomass	Reference
range of algal strains	Municipal Wastewater	4.9-11.3	-	Woertz et al 2009
<i>C. vulgaris</i>	Secondary municipal wastewater effluent	0.01	-	De-Bashan et al 2002
<i>C. vulgaris</i>	Nutrient replete medium	18.4	-	Rodolfi et al 2009
<i>C. vulgaris</i>	Modified Fritzgerald medium	52	-	Widjaja et al 2009
<i>C. vulgaris</i>	BG-11 growth medium	-	56	Chen et al 2009
<i>C. vulgaris</i>	Bold's Basal medium	14.71	-	Converti et al 2009
<i>C. vulgaris</i>	-	20-30	-	Putt 2007
<i>C. vulgaris</i>	MBL	5.3	2	Harris 2010

Johnson and Sprague (1987) stated that algae lipids cannot actually be used in their raw form. Whereas algal lipids contain approximately 10 % oxygen, petroleum contains almost none. They add that about half of the triglycerides naturally convert to fatty acids when leaving the algae for duration of 16 to 24 h at 20°C.

Sheehan et al (1998) argued that bio-fuels are too viscous for today's diesel engines. They suggested that the triacylglycerols (TAGs) need to react with simple alcohols. This way alkyl ester, hence bio-diesel, can be produced.

Despite its cultivation in media water, research has shown that microalgae actually use less water than land crops (Li et al 2008). This is because the cells are grown in aqueous suspension; hence they have more efficient access to water (Widjaja et al 2009, Bott & Nayar 2008). For the same reason they perform well in taking up CO<sub>2</sub> and other nutrients.

Microalgae have a very high tolerance to the CO<sub>2</sub> content in its growth media. Some species can handle up to 15 % CO<sub>2</sub> concentration (Li et al 2008). They provide a high efficiency medium for CO<sub>2</sub> mitigation (Li et al 2008) and hence can possibly produce carbon-neutral fuel. This may be of particular interest for future carbon trading (Sheehan et al 1998, Pienkos 2007).

While some research of microalgae in wastewater has been conducted in recent years, the nutrient removal of ammonia-N, nitrate-N, nitrite-N, organic nitrogen and phosphate-P and dry weight of the harvested algae has rarely been reported. Table 2.3 shows the level of nutrient removal reported in the literature. Tsukahara & Sawayama (2005) grew *B. braunii* in a continuous batch system with secondary treated sludge as the growing medium. They fed the algae for 11 days and kept growing them without additional feed for an additional 19 days. During their test, *B. braunii* reduced nitrate from 7.67 mg/L to 0 mg/L within 6 days. Phosphorus was reduced from 0.02 mg/L to 0 mg/L within 1 day. Nitrite was also found to be consumed, but ammonium remained untouched. Algae growth was also tested in municipal wastewater and diluted dairy wastewater in a semi continuous reactor (Woertz et al 2009). These researchers utilised naturally occurring algae. They found that the ammonium-N removal and phosphate removal for the municipal wastewater was 84 to > 99 % and 93 to > 99 % respectively. The differences in removal efficiency were due to different hydraulic retention times along with CO<sub>2</sub> and air feeding. They also achieved a 96 % ammonium removal and a > 99 % orthophosphates removal for the diluted dairy wastewater. The growth period was between 15 and 18 days. *Chlorella vulgaris* microalgae were also grown in urban wastewater using 3 L-bio-reactors (Ruiz-Marin 2009). These researchers found that the algae removed  $60.1 \pm 13.7$  % of ammonia-N after 50 hours. The nitrate-N removal was about 5 %. Park et al (2009) tested three different microalgae strains, including *Chlorella vulgaris* for 10 days. Their animal wastewaters included aerobic effluent and anaerobic effluent. For the aerobic and anaerobic effluent, total nitrogen was removed by about 38 % and total phosphorus was removed by about 24 %.

While nutrient removal is often reported in terms of ammonium-N and phosphate-P removal, little information is given for nitrate-N, nitrite-N and organic nitrogen. It is important to monitor the fate of all nitrogen forms in order to gain a complete picture of nutrient removal occurring in a bio-reactor.

TABLE 2.3 PREVIOUSLY RESEARCHED ALGAE GROWTH AND NUTRIENT REMOVAL IN DIFFERENT WASTEWATERS

Algal strain	Growth medium	Ammonia removal	Nitrate removal	Phosphate removal	Growth period	Reference
<i>B. braunii</i>	secondary treated sludge	0%	100%	100%	30 days	Tsukahara & Sawayama 2005
range of algal strains	municipal wastewater	84 - >99%	-	93 - >99%	15-18 days	Woertz et al 2009
range of algal strains	diluted dairy wastewater	96%	-	> 99%	15-18 days	Woertz et al 2009
<i>C. vulgaris</i>	urban wastewater	≈60.1%	≈5%	-	50 hrs	Ruiz-Marin 2009
<i>C. vulgaris</i>	diluted aerobic effluent	≈38% TN		≈24%	10 days	Park et al 2009
<i>C. vulgaris</i>	diluted anaerobic effluent	≈38% TN		≈24%	10 days	Park et al 2009

## 2.2 ADDITIONAL USES OF MICROALGAE BY-PRODUCTS

When microalgae are harvested and lipids are extracted, the remaining material can still be used as animal feed as a source of protein, carbohydrates and other nutrients (Chisti 2007). Alternatively anaerobic digestion can be used to produce methane (Chisti 2007).

## 2.3 MICROALGAE AS A VEHICLE FOR DECENTRALISATION

In recent years, there has been a push towards decentralisation as opposed to globalisation. Globalisation had a significant increase in importance from 1989 (Neef 2007). Since then, it has led to increased pollution and fuel demand due to an increase of long-haul transportation of goods and people, mass production of goods and a significant increase in energy demands. Globalisation also created the desire of more people to seek a western lifestyle. This leads to even more demand on fuel and energy, resulting in more pollution. Now that sustainability has taken on a large role in industry, politics and society, globalisation has started to make way to decentralisation. There has been a significant trend towards living, working,

manufacturing, producing, educating and shopping in the same small place (Neef 2007). Microalgae in particular can contribute to this new trend via decentralised wastewater polishing, bio-fuel cropping, carbon dioxide reduction as well as animal feed or methane gas production.

## 2.4 *CHLORELLA VULGARIS* AS A TYPICAL ALGAL STRAIN USED FOR CULTURING

*Chlorella vulgaris* (Figure 2.1) is an extensively researched alga. It belongs to the green freshwater microalgae strain (Widjaja et al 2009, Sheehan et al 1998). It consists of 87.4% moisture (Tsukahara & Sawayama 2005). *Chlorella vulgaris* microalgae have a lipid content of 20 – 30% of their dry weight (Putt 2007). This may be less than other algae strains, however, *Chlorella vulgaris* is extremely fast growing (see Table 2.4) and relatively robust (Widjaja et al 2009). Unfortunately *Chlorella vulgaris* can be very small with a diameter between 2-15  $\mu\text{m}$ , which can cause difficulties when harvesting the algae (Belcher & Swale 1978, Weber 1997).

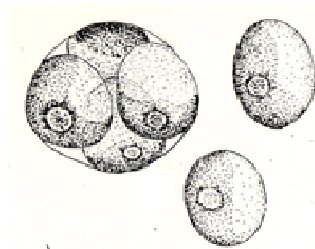


FIGURE 2.1 *CHLORELLA* (SOURCE: A BEGINNER'S GUIDE TO FRESHWATER ALGAE 1987)

*Chlorella vulgaris* requires high light intensity, warm temperatures of 20 to 26°C (Mata et al 2010), a pH of approximately 7.5 (Park et al 2009, Woertz et al 2009) and some trace elements such as iron (Park et al 2009). Since these conditions are also optimal for bacteria that achieve nutrient removal, it is important to keep the organic content of the wastewater measured in terms of 5-day BOD or COD at a minimum if algae need to be selectively grown. It was found that *Chlorella vulgaris* performed best in 250 mg/L COD piggy wastewater (Travieso 2006).

TABLE 2.4 DOUBLING TIMES AND LIPID CONTENTS OF DIFFERENT ALGAL STRAINS

Algal strain	Doubling time	Lipid content (% dry weight)	Reference
<i>C. vulgaris</i>	8.3 hrs	20-30	Posner & Sparrow 1964, Putt 2007
<i>B. braunii</i>	72 hrs	25-75	Sumukhi 2010, Li 2009

## 2.5 TYPICAL CULTURING TECHNIQUES

### 2.5.1 NUTRIENT REQUIREMENTS AND RATIOS

Chisti (2007) found that the essential elements for microalgae growth are nitrogen, phosphorous, iron and in some cases, silicon. However, Li (2009) did not note that iron was an essential element in the growth of microalgae. Chisti (2007) found that phosphorous must be supplied in excess, due to a low level of bioavailability. The phosphate ions were found to complex with metal ions in which cases the phosphorous would be unavailable. For a continuous growing set-up, the algae must be fed continuously during the daylight hours, but not during the night. Mixing however must occur at all times. The research also stated that up to 25 % of the produced biomass can be lost during the night time due to respiration. The respiratory impact depends on light levels, growing and night time temperature. Chisti (2007) found nitrate and phosphate fertiliser to be suitable for providing N and P.

Tsukahara and Sawayama (2005) grew *B. braunii*, which readily consumed nitrate and nitrite, but not ammonia. But the research found that *Chlorella vulgaris* used ammonia as a nitrogen source. They also discovered that *Chlorella vulgaris* feeds on organic and inorganic nitrogen sources.

Widjaja et al (2009) used Sodium nitrate ( $\text{NaNO}_3$ ) as a nitrogen source and large amounts of Dipotassium phosphate ( $\text{K}_2\text{HPO}_4$ ) and small amounts of Potassium phosphate ( $\text{KH}_2\text{PO}_4$ ) as a phosphorous source. Miyamoto (1997) reported the use of Potassium nitrate ( $\text{KNO}_3$ ) for nitrogen and the same solutions as Widjaja et al (2009) for phosphorous sources.

$\text{KNO}_3$  and  $\text{KH}_2\text{PO}_4$  have been used as a nitrogen source and phosphorous source respectively by many scientists in the past (Pratt & Fong 1940, Krauss 1953, Gummert et al 1953, Fogg & Collyer 1953).

Most algae require substrate in N:P:C ratio of 8:1:50 (Lundquist 2006). Other authors used the Redfield N:P:C ratio of 16:1:106 (Grobelaar 2004). The carbon component is very high and hence needs to be enriched by  $\text{CO}_2$  to achieve optimal growth conditions. Lundquist (2008) suggested that artificial  $\text{CO}_2$  addition significantly improves nutrient removal and growth rate of algae. However, he added that the ideal N:P:C ratio has not been found yet.

### 2.5.2 CARBON DIOXIDE AND pH REQUIREMENTS

pH is known to rise as algae consume  $\text{CO}_2$  (see Section 2.7) and reduce its concentration (Chisti 2007). To avoid a drastic pH rise, pH should be monitored at all times and  $\text{CO}_2$  should be fed accordingly. Widjaja et al (2009) suggested that *Chlorella vulgaris* can survive in low pH, however the growth also is reduced. They recommended a pH range of 5 to above 8. Woertz et al (2009) recommended a pH range of 7-8. Park et al (2009) suggested a pH range of 7.5 to 8.5.

A high proportion of algae are made up of carbon. The majority of this carbon is sourced from carbon dioxide. Chisti (2007) found that it was important that carbon dioxide was continually available during daylight hours to ensure optimal growth. He found that 100 tons of algal biomass consumes 183 tons of carbon dioxide. Li (2009) found the algae to  $\text{CO}_2$  ratio to be 10:17. Lundquist (2008) found that  $\text{CO}_2$  addition significantly improved nutrient removal and algae growth rate.

Krauss (1953) found that *Chlorella* is unable to take up bicarbonate as a carbon source. He believed the reason for that may be that the bicarbonate ions or the undissociated salts cannot pass through the algae's membrane.

### 2.5.3 *LIGHT REQUIREMENTS*

Light is the most important limiting factor in algae growing (Mata et al 2010). Chisti (2007) found that some algae varieties have a light saturation constant of 185-200  $\mu\text{E}/\text{m}^2\text{s}$ . The light saturation constant refers to the light saturation at half of the algae's maximum growth rate. Immediately after the maximum growth rate peak at a certain light intensity, the photoinhibition-zone begins where the growth rate decreases with increasing light intensity. Overcoming this light saturation problem is still part of extensive research (Sheehan et al 1998). An Amsterdam based research group has found a way to theoretically overcome light saturation (Benemann & Oswald 1994).

Chisti (2007) also mentioned the possibility of achieving higher biomass productivity when light-dark cycles are introduced. He stated that 10 ms cycles have proven to result in a higher biomass growth rate. However the ideal frequency of light-dark cycles has not been identified yet.

Sheehan et al (1998) suggested light and dark cycles of 1 second each to achieve the so-called “flashing light effect”, a more efficient utilisation of light. However, they also mentioned that this particular frequency of flashing light had only marginal effects on the growth rate of algae.

Phillips & Myers (1953) noted that based on theoretical models of photosynthesis, plants can make most efficient use of high intensity light when it comes in flashes. They recommended 1 ms flashes separated by long dark periods. They suggested that even 67 ms would perform satisfactory. The dark periods should be in the order of ten times the flash period.

Gonzalez et al (2008) used a light intensity of 4500 lux, where Ruiz-Marin et al (2009) suggested a light intensity of 135  $\mu\text{E}/\text{m}^2\text{s}$ . Park et al (2009) grew their algae under 12 hour light and 12 hour dark conditions at 35-40  $\mu\text{E}/\text{m}^2\text{s}$ . Kebede-Westhead et al (2006) used two 400 Watt metal halide lights for 23 h with 1 h darkness. Silva et al (2009) used continuous illumination with 6000-10000 lux fluorescent light.



Fogg & Collyer (1953) found that there were no differences in yield if dark and light periods were alternated. But they found that the algae stopped growing during dark periods and started growing exponentially as soon as the light period started again.

#### 2.5.4 TEMPERATURE REQUIREMENTS

Temperature is the second most important limiting factor for algae growth (Mata et al 2010). Mata et al (2010) and Moheimani (2005) stated that most algae strains can tolerate up to 15°C below their optimum temperature, but the growth can be severely inhibited at a temperature rise of 2-4°C above the optimum. They suggest a constant temperature between 20 to 26°C.

Woertz et al (2009) recommended a growing temperature of 23 to 25°C. However, Gonzalez et al (2008) proposed a temperature of 30°C. Ruiz-Marin et al (2009) and Park et al (2009) suggested  $25 \pm 1^\circ\text{C}$ . Kebede-Westhead et al (2006) maintained a temperature of 23-26°C in their experiment and Silva et al (2009) used a temperature of 21-24°C. Brennan & Owende (2010) suggested 25°C for *Chlorella vulgaris*.

#### 2.5.5 ORGANIC LOAD TOLERANCES

Benemann and Oswald (1994) mentioned that at the Sunnyvale (California) oxidation ponds facility, *Chlorella* algae appeared naturally during the winter months. They suggested that this can be due to *Chlorella*'s tolerance to lower pH and higher BOD compared to other algae species. However, it was not stated exactly how much BOD they can handle.

Travieso et al (2006) found that *Chlorella vulgaris* performed best in 250 mg/L COD (equivalent to 100-150 mg/L BOD) piggery wastewater.

### 2.5.6 MIXING TECHNIQUES

In batch cultures, mixing is an essential part of growing algae. It provides with evenly distributed cells, nutrients, heat, gases and metabolites (Mata et al 2010). It is however important to note that a certain degree of turbulence in the water can result in shearing and death of the algae (Mata et al 2010, Moheimani 2005). Turbulence occurs due to mechanical mixing and gas bubbles.

### 2.5.7 ALGAE RECOVERY AND HARVESTING

The recovery of microalgae is often difficult and hence expensive due to the algae's small size (2-20  $\mu\text{m}$ ) and concentration (500 ppm) (Li 2009). Chisti (2007) suggested algae recovery through broth filtration or centrifugal drying (Park et al 2009). Li (2009) also suggested flocculation and froth flotation. He mentioned harvest chemicals such as alum and ferric chloride. For *Chlorella* algae, he suggested cellulose fibre addition at a rate of 10 % algae weight with a static mixer, then ferric nitrate addition and finally dewatering.

Widjaja et al (2009) harvested their cells through centrifugation at 8500 rpm for 5 min and washed once with distilled water followed by freeze drying. Ruiz-Marin et al (2009) suggested a cell harvest by centrifugation at 3500 rpm for 10 min. Kebede-Westhead et al (2006) harvested with wet/dry vacuum and dewatered by a 3mm sieve, followed by overnight drying with a fan at 25°C. Brennan & Owende (2010) said that centrifuging can achieve a harvesting efficiency of more than 95 % with a total of up to 15 % suspended solids in the slurry. They added that it is important to dry the slurry quickly. They suggest sun drying, low-pressure shelf drying, spray drying, drum drying, fluidised bed drying, freeze drying and a drying technology referred to as Refractance Window. They recommended freeze drying because it eases the extraction of oils through less cell disruption when solvents are used.

## 2.6 MICROALGAE GROWTH PHASES

Algae undergo five growth phases as shown in Figure 2.2. These are the lag phase, the exponential phase, the linear growth phase, the stationary growth phase and the death phase.

At first the algae are likely to experience some shock when transferred from the stock solution to the growth solution. During that time, the algae will grow very little or not at all, hence it is referred to as the lag phase. The phase can last up to a few days. The shock may be due to non-viable cells or spores in the growth medium, changed culture conditions or change in nutrient levels (Lee & Shen 2004). The phase is overcome as soon as adjusted cells are used as inoculums (Lee & Shen 2004).

When the cells have adjusted to the new conditions, they enter an accelerated growth phase where they grow and divide. If enough nutrition and light is provided in excess, the algae start growing exponentially (Lee & Shen 2004).

As the algae cell concentration increases, the quantity of light energy absorbed reduces and the algae enters a linear growth rate. The phase remains until nutrients or light energy become the limiting factor or if some inhibitors develop (Lee & Shen 2004). At that point, the death phase is present.

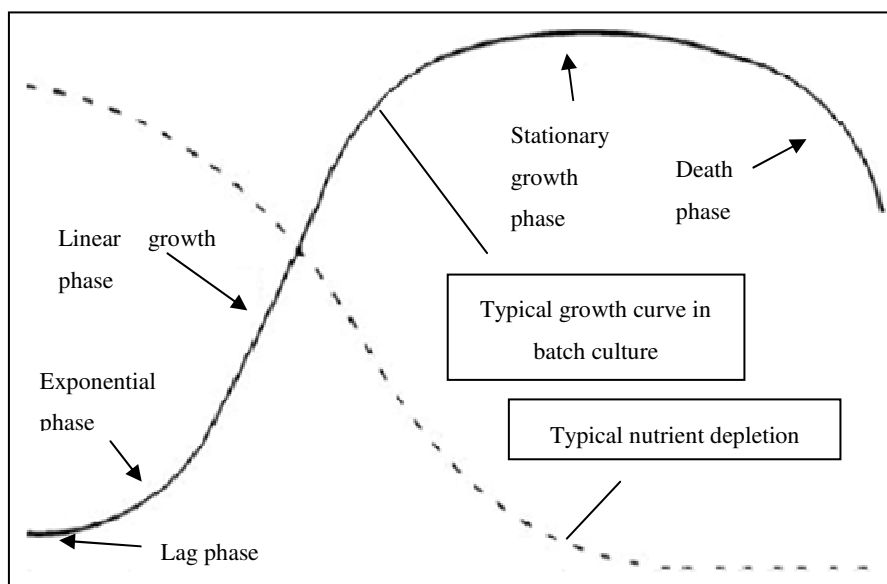


FIGURE 2.2 MICROALGAE UNDERGO TYPICAL GROWTH PHASES BASED ON NUTRIENT AND LIGHT AVAILABILITY AND OTHER LIMITING GROWTH FACTORS.

## 2.7 PHOTOSYNTHESIS IN MICROALGAE

Photosynthesis is the main engine driving CO<sub>2</sub> mitigation, production of O<sub>2</sub>, algal growth and pH changes. This section gives an insight of photosynthesis in order to gain a better understanding of the processes involved.

Photosynthesis consists of two cycles, the light and dark reaction. The light reaction takes place in the chloroplast's grana. Water can split and produce OH<sup>-</sup> and H<sup>+</sup> ions. Low energy electrons become available through splitting of hydroxyl ions (OH<sup>-</sup>) to one electron and one OH molecule. The OH molecules then combine to water and oxygen molecules. In the algal cell, a group of chlorophyll molecules act as light traps. A special chlorophyll molecule absorbs light energy and ejects a high-energy electron through taking up the low energy electron from the hydroxyl ion split. The high-energy electron is then absorbed by another specialised pigment, which emits a new electron of slightly less energy. This particular process continues for a number of times and hence electrons are transported down a potential energy gradient, the "energy transport chain". Some of the energy lost by the electrons travelling down the energy gradient is fixed in the form of an energy-carrying molecule "ATP" (adenosine triphosphate). Some electrons then go through a second chlorophyll light trap to reduce another energy carrying molecule "NADP" (nicotinamide adenine dinucleotide).

phosphate) to “NADPH” (nicotinamide adenine dinucleotide phosphate). The remaining electrons go through the electron transport chain again to produce more ATP. NADPH is produced with the use of the hydrogen ion from the water and hence neutralising the withdrawal of the  $\text{OH}^-$  earlier. The hydroxyl groups that lost electrons to the chlorophyll combine in pairs to produce water and oxygen gas (Forbes & Watson 1992). The conversion of light energy to chemical energy is shown in Figure 2.3.

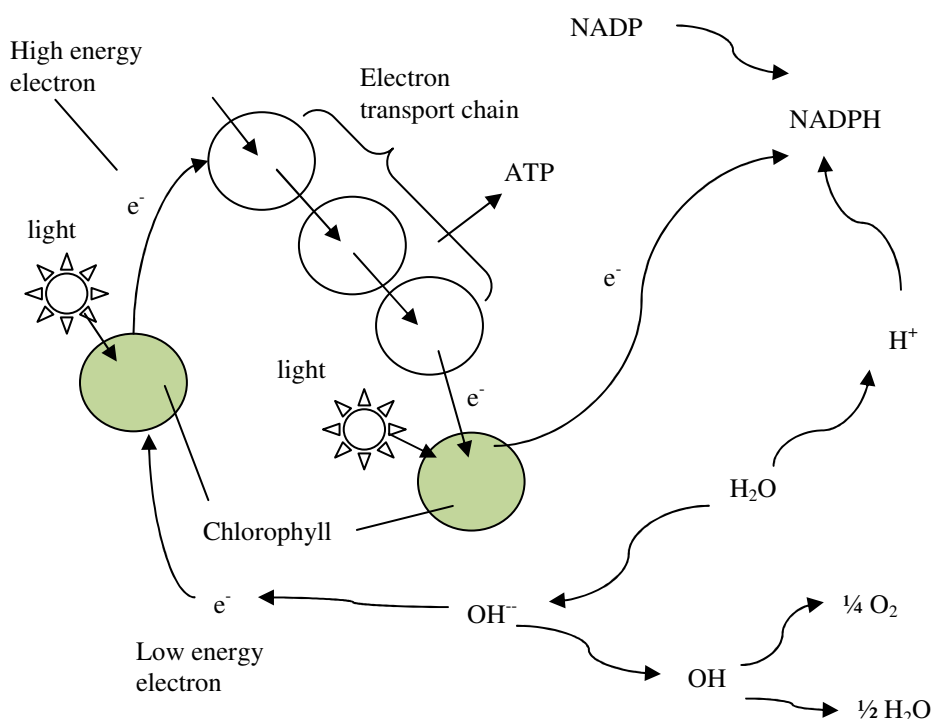
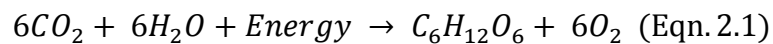


FIGURE 2.3 CONVERSION OF LIGHT ENERGY TO CHEMICAL ENERGY IN THE LIGHT REACTION (ADOPTED FROM FORBES & WATSON 1992)

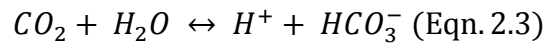
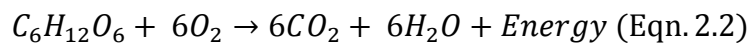
After the light reaction, the dark reaction or the “Calvin cycle” follows. The dark reaction occurs in the stroma of the chloroplast. ATP and NADPH provide energy to change carbon dioxide to carbohydrates via the Calvin Cycle, the “carbon dioxide assimilation”. This means that carbon dioxide is fixed into organic compounds. This is initiated by the chemical RuBP (Ribulose biphosphate), which combined with carbon dioxide produces two 3-carbon-products. This is further reduced to PGAL and then finally produces  $\text{C}_6$  sugar. One  $\text{C}_6$  sugar requires 6  $\text{CO}_2$ , 12 NADPH, 18 ATP and 6 water molecules are generated. Some of the PGAL molecules continue through the Calvin cycle in a number of chemical reactions to produce new RuBP (Forbes & Watson 1992).

Carbohydrates are mainly transported around the plant as sucrose. Carbohydrate compounds are required for algal growth and metabolism.

The overall equation for photosynthesis is shown in Equation 2.1. It shows how plants synthesise carbohydrates using light energy, water and carbon dioxide. As light energy is required for this process, photosynthesis only occurs during light periods (Forbes & Watson 1992).



The process of cellular respiration, as shown in Equation 2.2, occurs simultaneously. Respiration occurs during light and dark cycles. During this process, carbon dioxide is released into the water, resulting in the formation of carbonic acid and hence decreasing the pH as shown in Equation 2.3 (Gregory 2006).



When sufficient light is available during the light period,  $CO_2$  mitigation dominates  $CO_2$  release. However, during the dark period, algae take up oxygen and produce carbon dioxide like other organisms in the water body. This can result in a substantial decrease in available oxygen in the water, especially in the hours before dawn. It is therefore a necessity to reduce the risk of algae outbreaks in nature.

It is important to note that photosynthesis only uses light energy, water and  $CO_2$  to produce simple sugars, oxygen and water. Algae and all other plants also synthesise other, more complex compounds with the use of sugars produced during photosynthesis. Nitrogen is involved in some of these processes (e.g. in the production of amino acids), but not with photosynthesis itself (Bassham 2010). The same applies for other nutrients such as phosphorous.

## 2.8 CHAPTER SUMMARY

This chapter reviewed the literature relevant to microalgae uses as bio-fuel, wastewater polisher, CO<sub>2</sub> sink and a way to apply these beneficial uses to decentralisation. Data from previous research conducted on nutrient removal and lipid production during algae production was shown. A number of culturing techniques were outlined and compared. These techniques were adjusted for this experiment as shown in Chapter 3. In order to explain the experimental results in Chapters 4 to 7, different microalgal growth phases as well as photosynthesis were explained.

## CHAPTER 3      METHODOLOGY

Identifying the level of nutrient removal and lipid production with *Chlorella vulgaris* microalgae in different wastewaters consisted of a number of steps:

1. Collection and preparation of wastewater
2. Determination of the wastewater characteristics
3. Mixing and possible nutrient addition to the wastewater
4. Online monitoring of pH, DO and temperature in the reactor
5. Samples collection for dissolved solids, spectroscopy, nitrogen and phosphorous measurements (nutrient and growth data) as well as recording water volume in the reactor
6. Harvesting of algae and freeze drying
7. Lipid determination and extraction
8. Analysis of online data, nutrient and growth data and lipid information

### 3.1 WASTEWATER COLLECTION AND PREPARATION

#### 3.1.1 NURSERY RUNOFF

The nursery runoff water was collected from the iceberg and cos-lettuce nursery at Story Fresh, Cambooya. The wastewater was collected using a 30 mm diameter hose which was connected to the drain of the nursery during the fertilizer application. The fertilizer was applied via overhead irrigation. The majority of water collected drained through the seedling pots into the drain. The seeding pots contained some additional fertilizer and soil media amendments such as lime, wetting agents, rock phosphate, trace elements and NPK fertilizers. The first and second dairy wastewater batch was collected on 8 August, 2010 and 9 September, 2010 respectively.



### 3.1.2 LETTUCE FACTORY WASTEWATER

The lettuce factory wastewater was collected from the lettuce processing facility at Story Fresh, Cambooya. The wastewater was collected during the factory's mid-day cleaning procedure, which resulted in a wastewater containing lettuce waste, soil and chlorine based chemicals. Wastewater was collected at the drain of the factory. The water for the first and second batch experiment was collected on 16 April, 2010 and 14 May, 2010 respectively.

### 3.1.3 MUNICIPAL WASTEWATER

The municipal wastewater was collected at the Wetalla wastewater reclamation facility at Harlaxton, Toowoomba. The collected wastewater included raw wastewater influent at the inlet of the reclamation facility and effluent from the clarifier. The wastewater for the first and second batch experiment was collected on 22 July, 2010 and 26 August, 2010 respectively.

### 3.1.4 DAIRY WASTEWATER

The dairy wastewater was collected from the Sunnymount dairy farm, Cambooya. The wastewater was pumped from the bottom of a lagoon. The primary use of this water is cleaning the milk station. According to the dairy farmer, chemicals such as hydrochloric acid, iodine, sodium hydroxide and antibiotics may be present in the water. The wastewater for the first and second batch experiment was collected on 09 August, 2010 and 13 September, 2010 respectively.

### 3.1.5 PREPARATION AND STORAGE

On the day of the wastewater collection, the water was filtered using 0.45  $\mu\text{m}$  filter paper using a vacuum pump and then stored at 4°C for up to one week.

## 3.2 WASTEWATER CHARACTERISTICS

Raw wastewater characteristics were tested for organic strength and nutrients in order to gauge their concentrations for adequate growth conditions needed for *Chlorella vulgaris*.

Organic strength of the wastewater was measured both by 5-day BOD according to Standard Methods (APHA 1995) as well as by total organic carbon (TOC) using Total Organic Carbon/ Total Nitrogen Analyzer (TOC-VCPH/CPN) (see Figure 3.1). Nutrients present in the wastewater such as nitrate nitrogen, nitrite nitrogen and phosphate phosphorous in the liquid phase were measured using Ion Chromatography system (IC, Dionex ICS 2000) using an anion (AS-18) column during the analytical process.



FIGURE 3.1 TOTAL ORGANIC CARBON/ TOTAL NITROGEN ANALYZER (TOC-VCPH/CPN) TESTING A SAMPLE

For every wastewater batch, water was collected from one to three different sources (see Section 3.1). To achieve a suitable mix of these wastewater sources to maximise algae growth in the selected wastewater BOD, TOC, TN and  $\text{PO}_4\text{-P}$  of the different sources were compared. Figure 3.2 shows an example how such a comparison was conducted. Each ‘part’ was equal to 500 ml of wastewater. According to different concentrations of the parts, the total amount of nutrients and organic strength was calculated for 3.5 L medium. The aim was to achieve a N:P ratio of 8-16:1 (see Section 2.5.1). The desired 5-day BOD was below 150 mg/L. Comments were made accordingly and the most suited option of the wastewater mix

was chosen. For example, for the experiment with municipal wastewater, samples collected from inlet and clarifier had the concentrations of 280.9 mg TN/L, 69.5 mg TN/L, 6.5 mg P/L, 0.3 mg P/L, 238.2 mg TOC/L, 44.9 mg TOC/L, 167.5 mg BOD/L and 2mg BOD/L respectively (see Figure 3.2) respectively. By adjusting the volumes of the two wastewater sources, the desired concentration of 129.9 mg TN/L, 2 mg P/L, 100.2 mg TOC/L and 49.3 mg BOD/L (see option 2 in Figure 3.2) was achieved.

Mixes		
	Clari	Real
TN (mg/L)	69.475	280.9
P (mg/L)	0.2605	6.469
TOC (mg/L)	44.93	238.2
BOD (mg/L)	2	167.5

Option 1		
	Parts	
	6	1

	Amounts		Total	TN-P Ratio	Comment
TN (mg/L)	59.550	40.129	99.679	86.9	needs adjustment
P (mg/L)	0.223	0.924	1.147	1	
TOC (mg/L)	38.511	34.029	72.540		good
BOD (mg/L)	1.714	23.929	25.643		good

Option 2		
	Parts	
	5	2

	Amounts		Total	TN-P Ratio	Comment
TN (mg/L)	49.625	80.257	129.882	63.8	needs adjustment
P (mg/L)	0.186	1.848	2.034	1	
TOC (mg/L)	32.093	68.057	100.150		good
BOD (mg/L)	1.429	47.857	49.286		good

Option 3		
	Parts	
	4	3

	Amounts		Total	TN-P Ratio	Comment
TN (mg/L)	39.700	120.386	160.086	54.8	needs adjustment
P (mg/L)	0.149	2.772	2.921	1	
TOC (mg/L)	25.674	102.086	127.760		ok
BOD (mg/L)	1.143	71.786	72.929		good

FIGURE 3.2 SAMPLE CALCULATION FOR PREPARING THE WASTEWATER MEDIA

### 3.2.1 SPECIAL ADJUSTMENTS TO THE WASTEWATER MEDIA

In order to adjust the nutrient ratio or the organic strength, the wastewater had nutrients added or was diluted with distilled water. For the first test of municipal wastewater, an adjustment of the nutrient ratio was required. In order to do that, 50 ml of  $K_2HPO_4$  was added to the bio-reactor. A sample calculation for the strength of  $K_2HPO_4$  can be found below.

Concentration of TN in the bio-reactor = 129.882 mg/L (see option 2 in Figure 3.2)

Concentration of P in the bio-reactor = 2.034 mg/L

$$\text{Current } N:P \text{ ratio} = \frac{TN}{P} = 63.8 \text{ (Eqn. 3.1)}$$

Desired N:P ratio = 8:1

To achieve this ratio, 16.235 mg/L of P are required as shown in Eqn. 3.2.

$$\text{mg/L concentration of P required} = \frac{TN}{8} = 16.235 \frac{mg}{L} \text{ (Eqn. 3.2)}$$

As there are 3.5 liters in the bio-reactor, the total amount of P required is 3.5 L x (16.235-2.034) mg/L, therefore 49.704 mgP. In order to add only 50 ml of the nutrient solution to the bio-reactor, the following calculations were undertaken:

molecular weight of  $K_2HPO_4$  = 174 g/mol

molecular weight of P = 31 g/mol

$$\begin{aligned} \text{concentration of P required for 50 ml sample} &= \frac{49.704 \text{ mgP}}{50 \text{ ml}} \\ &= 0.994 \frac{\text{mgP}}{\text{ml}} \text{ (Eqn. 3.3)} \end{aligned}$$

It follows that,

$$\begin{aligned} \text{concentration of } K_2HPO_4 \text{ in sample} &= 0.994 \frac{mgP}{ml} * \frac{174 g K_2HPO_4}{31 g P} \\ &= 5.580 \frac{mg K_2HPO_4}{ml} = 5.580 \frac{g K_2HPO_4}{L} \quad (\text{Eqn. 3.4}) \end{aligned}$$

50 ml of the  $5.580 \frac{g K_2HPO_4}{L}$  were then inoculated into the bio-reactor.

For the first test of dairy wastewater, the nutrient ratio also required adjustment. In order to do that, 50 ml of  $NaNO_3$  was added to the bio-reactor. A sample calculation for the strength of  $NaNO_3$  can be found below. The dairy water was also diluted with 2.5 L distilled water (see Section 6.1). To avoid an unnecessary increase of water in the reactor through the addition of nitrogen, only 2.45 L of distilled water were used for dilution. The remaining 50 ml were topped up with the  $NaNO_3$  solution.

Concentration of TN in the bio-reactor = 105.486 mg/L

Concentration of N in the bio-reactor = 13.868 mg/L

$$N:P \text{ ratio} = \frac{TN}{P} = 7.61 \quad (\text{Eqn. 3.5})$$

Desired N:P ratio = 10:1

To achieve this ratio, 138.68 mg/L of N are required as shown in Eqn. 3.5.

$$mg/L \text{ concentration of } N \text{ required} = 10 * P = 138.68 mg/L$$

As there are 3.5 liters in the bio-reactor, the total amount of N required is 3.5 L x (138.68-105.486) mg/L, therefore 116.179 mg N. In order to add only 50 ml of the nutrient solution to the bio-reactor, the following calculations were undertaken:

molecular weight of  $\text{NaNO}_3 = 85 \text{ g/mol}$

molecular weight of  $\text{N} = 14 \text{ g/mol}$

$$\begin{aligned} \text{concentration of N required for 50 ml sample} &= \frac{116.179 \text{ mgN}}{50 \text{ ml}} \\ &= 2.324 \frac{\text{mgN}}{\text{ml}} \quad (\text{Eqn. 3.6}) \end{aligned}$$

It follows that,

$$\begin{aligned} \text{concentration of NaNO}_3 \text{ in sample} &= 2.324 \frac{\text{mgP}}{\text{ml}} * \frac{85 \text{ g NaNO}_3}{14 \text{ g N}} \\ &= 14.107 \frac{\text{mg NaNO}_3}{\text{ml}} = 14.107 \frac{\text{g NaNO}_3}{\text{L}} \quad (\text{Eqn. 3.7}) \end{aligned}$$

50ml of the  $14.107 \frac{\text{g NaNO}_3}{\text{L}}$  were then inoculated into the bio-reactor.

### 3.3 BIO-REACTOR DESIGN

A microalgae based titrimetric bio-reactor was installed in the Environmental (water and wastewater) laboratory, Faculty of Engineering and Surveying, University of Southern Queensland that enabled the real time data collection corresponding to the growth of *Chlorella vulgaris* microalgae (Figure 3.3 & Figure 3.4). The batch study was conducted using a single reactor having a capacity of 3.5 liters. Compressed air was supplied continuously at 250 ml/min for correct aeration and an overhead stirrer was provided with the reactor in order to mix the content uniformly.  $\text{CO}_2$  was fed continuously at a rate of 10 ml/min from the bottom of the reactor. When the microalgae was adjusted to the new conditions and growth was established (after about 4 days),  $\text{CO}_2$  feeding occurred every two hours for 15 seconds during the light-period at a flow of 56 ml/min. Some wastewaters with less buffering capacity or a naturally low pH received a reduced  $\text{CO}_2$  feed of 5-10 seconds. More information regarding reduced feeding times can be found in Chapters 5 and 6. Chisti (2007) found that it was important that carbon dioxide was continually available during

daylight hours to ensure optimal growth. There were two florescent light sources (2000 lux each) 10 cm from the reactor to provide the required light intensity. Light was supplied for duration of 16 hours starting from 5 am to 9 pm.

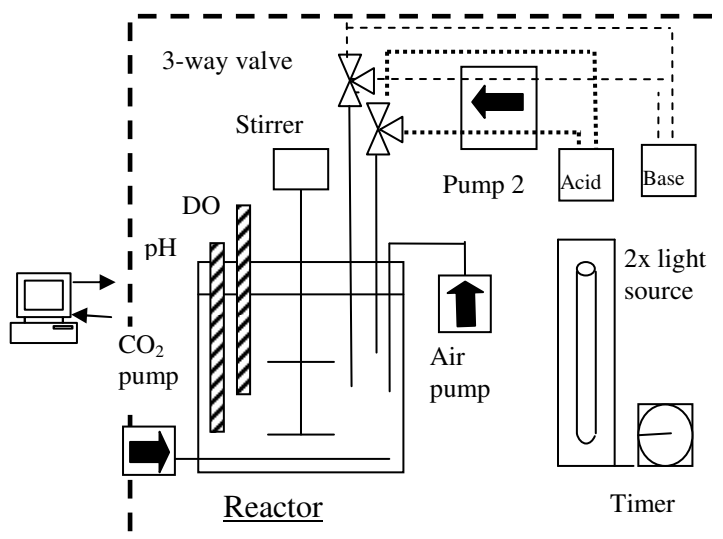


FIGURE 3.3 SCHEMATIC DIAGRAM OF THE BIO-REACTOR

A titrimetric unit, consisting of Ionode pH electrode connected with the pH transmitter (TSP Mini Chem), two 3- way solenoid valves, an acid tank and a base tank, were installed in order to monitor and control the pH of the system during the experimental run. The acid and base were continuously pumped around by a peristaltic pump to keep a constant liquid pressure in the dosage system and to maintain constant dose rate. The data acquisition unit transmitted the signals to the computer equipped with the Labview software package (National Instruments). In addition, the reactor was assembled with a dissolved oxygen electrode (YSI). The Labview software was used for monitoring the dissolved oxygen as well as temperature serial output from dissolved oxygen meter (TPS 90-D) and pH data with high frequency. The Labview package also controlled both of the 3-way solenoid valves that were assembled in the titrimetric respirometer for acid and base pulsing respectively to keep the pH in the reactor constant. The 0-1 volt signals from the transmitter were logged by a PC equipped with the Labview software package and a combined A/D I/O card (National Instruments, PCI-6013). All data acquired from the experiment were recorded in a Microsoft Excel spread sheet format. The user could set the parameters on the front panel with the

tolerance set- point limit. During the batch experiments, both pH and DO profiles were monitored every minute and pH was commonly controlled at a set point of  $7.5 \pm 0.3$  by automatic addition of base (0.1 N NaOH) or acid (0.05 N H<sub>2</sub>SO<sub>4</sub>) solutions with two 3-way solenoid valves. For wastewaters with a smaller buffering capacity or a naturally low pH, the pH set point range was increased to 0.5 to 0.6. More information regarding changed set point ranges is provided in Chapters 5 and 6. Temperature was controlled in the laboratory using the air conditioning system at 25°C. However, the reactor temperature was found to fluctuate between 18 and 24°C. Therefore, a temperature correction was performed on the DO data to a base of 20°C to maintain the consistency.

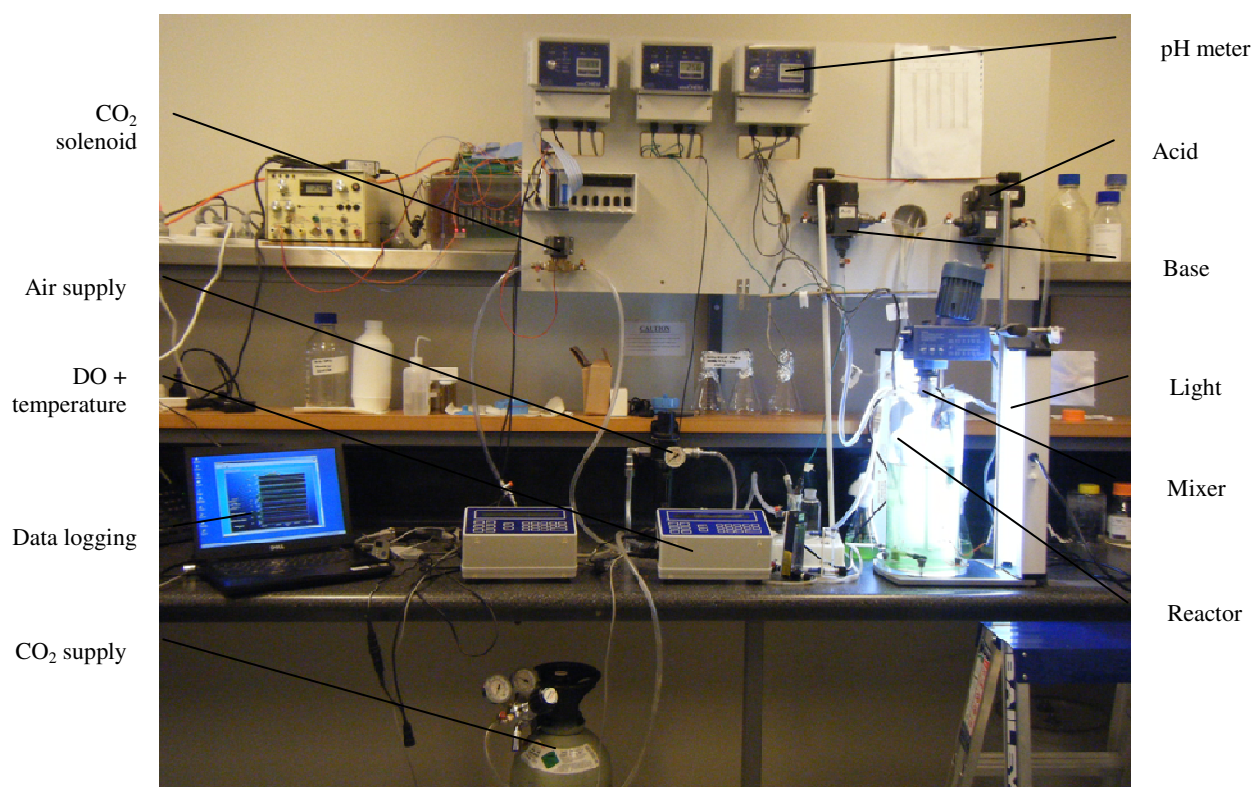


FIGURE 3.4 EXPERIMENTAL SET-UP



### 3.4 PRE-CULTURING AND INOCULATION OF *CHLORELLA VULGARIS*

*Chlorella vulgaris* was pre-cultured in MBL and then inoculated into the bio-reactor. The composition of MBL and the pre-culturing technique is described below.

#### 3.4.1 PREPARATION OF MBL

MBL medium was prepared as shown in Table 3.1 (Nichols 1973).

TABLE 3.1 COMPONENTS FOR THE PREPARATION OF MBL MEDIUM

Stock solutions	Per liter distilled/milliQ water	
CaCl <sub>2</sub> .2H <sub>2</sub> O	36.76 g	
MgSO <sub>4</sub> .7H <sub>2</sub> O	36.97 g	
NaHCO <sub>3</sub>	12.60 g	
K <sub>2</sub> HPO <sub>4</sub>	8.71 g	
NaNO <sub>3</sub>	85.01 g	
Na <sub>2</sub> SiO <sub>3</sub> .9H <sub>2</sub> O	28.42 g	
Na <sub>2</sub> EDTA	4.36 g	
FeCl <sub>3</sub> .6H <sub>2</sub> O	3.15 g	
Metal Mix		Add each separately to 750 mL, fully dissolved between additions and then increase volume to 1 liter with distilled water.
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.01 g	
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.022 g	
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.01 g	
MnCl <sub>2</sub> .4H <sub>2</sub> O	0.18 g	
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.006 g	
Vitamin stock		
Cyanocobalamin (Vitamin B12)	0.0005 g/L	
Thiamine HCl (Vitamin B1)	0.10 g/L	
Biotin	0.0005 g/L	
Tris stock	250.00 g/L	

The stock solutions were stored at 4°C. To make up the MBL medium, 1 ml of each stock solution was added to 1 L milliQ water. The pH was then adjusted to 7.2 with HCl, followed by autoclaving at 121°C and 15 PSI for 15 minutes. The MBL medium was stored at 4°C for up to three months.

### 3.4.2 PRE-CULTURING OF *CHLORELLA VULGARIS*

*Chlorella vulgaris* was initially grown in 250 ml Erlenmeyer flasks (Figure 3.5) with 150 ml MBL and 15 ml *Chlorella vulgaris* in MBL from an older culture (usually 3 to 4 weeks old culture). The flasks and cotton buds were autoclaved to reduce the risk of contamination through other microorganisms. The *Chlorella vulgaris* cultures were kept in the same room and at the same temperature as the bio-reactor. In addition, they underwent the same dark and light periods as the batch experiment. This was done to aid acclimatisation of the algae.



FIGURE 3.5 YOUNG AND OLDER CULTURE OF *CHLORELLA VULGARIS* IN MBL IN ERLLENMEYER FLASKS WITH COTTON BUDS

### 3.4.3 INOCULATION OF *CHLORELLA VULGARIS* INTO THE BIO-REACTOR

The entire content in the Erlenmeyer flask was used to inoculate the bio-reactor. This included *Chlorella vulgaris* microalgae and their growth medium – MBL. It is important to note that the composition of the MBL would have been different compared to its initial composition, as the algae would have utilised some of the nutrients and vitamins in the growth medium while in the Erlenmeyer flask.

### 3.5 EXPERIMENTAL MEASUREMENTS

#### 3.5.1 MEASUREMENT OF ALGAL GROWTH

The algal growth was measured in terms of suspended solids and by quantifying the cell density using spectroscopy (Figure 3.6). Suspended solids were measured using Standard Methods (APHA 1995). Measurements were undertaken over four consecutive days per week. Later in the experiment the suspended solids measurement period was increased to five consecutive days per week. At the same time, pH and DO profiles were also automatically logged into the system that gives an indication of growth in real-time.

For the spectrometry (Jenway 6705 UV/Vis. Spectrophotometer), the wavelength was set at 505 nm. The baseline was determined with the filtrate from the 0.45  $\mu\text{m}$  suspended solids filtration. Spectrometry measurements were taken over 5 consecutive days per week.

In addition, the filter papers from the suspended solids measurements were collected as a visual guide of growth. These filter papers will be shown in Chapter 4 to 6.

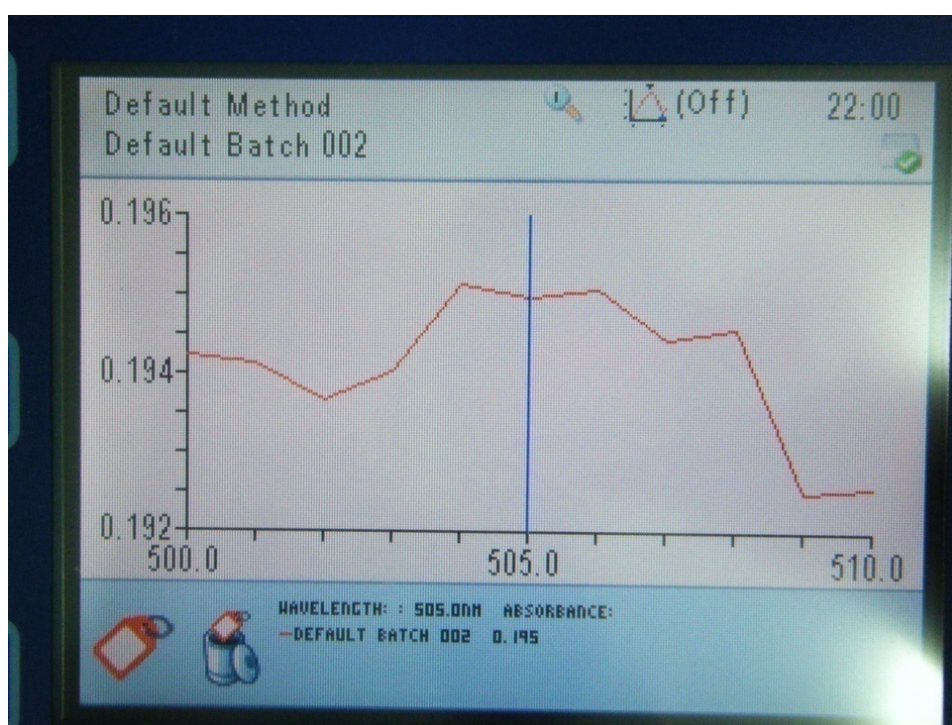


FIGURE 3.6 DATA OUTPUT BY THE JENWAY 6705 UV/VIS. SPECTROPHOTOMETER

### 3.5.2 MEASUREMENT OF NUTRIENT DEPLETION

The batch experiments were conducted for 12 to 18 days. During this time, the liquid samples amounting to 20 ml were collected from the reactor each day for 5 days per week and filtered for nutrient analysis. Prior to sampling, the microalgae were put in suspension through bubbling air vigorously and overhead mixing to achieve complete mixing and to ensure achieving a representative sampling for measurement purposes. The batch experiments were terminated when the death phase was ensured which was found to start after 10 to 15 days post inoculation of *Chlorella vulgaris* into the bio-reactor. Nutrient depletion was measured as described in Section 3.2. Figure 3.7 shows samples prepared for nutrient measurement. Specific growth rate was calculated according to Section 3.6.2.

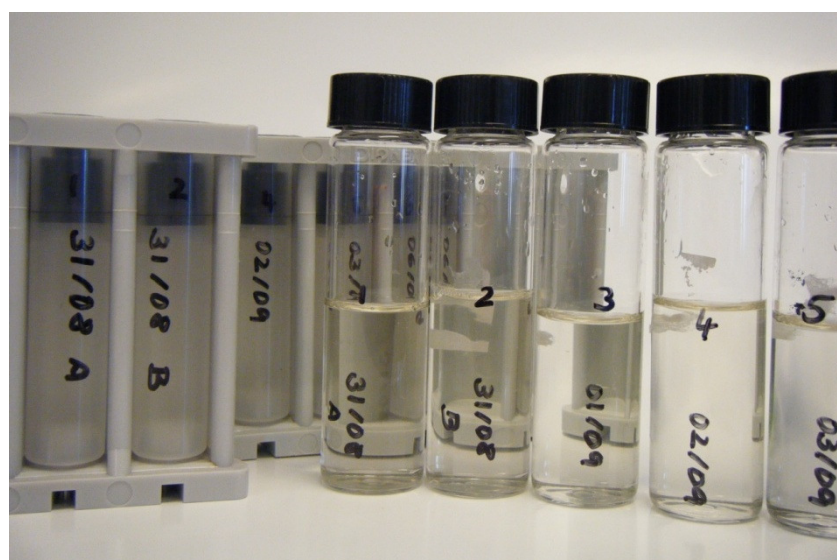


FIGURE 3.7 5ML SAMPLES FOR ION CHROMATOGRAPHY SYSTEM (IC, DIONEX ICS 2000) MEASUREMENTS (LEFT) AND  $\approx$ 15ML SAMPLES FOR NITROGEN ANALYSER (TOC-VCPH/CPN) MEASUREMENTS (RIGHT)

### 3.5.3 MEASUREMENT OF ONLINE DO AND pH DATA

The reactor was equipped with a DO and pH meter as described in Section 3.3. To assure the most accurate data achievable, both probes were calibrated at least once per week. Data from the pH probe and the DO probe was fed to the Labview software in 1 minute intervals. The data was saved on a Microsoft Excel spread sheet and was also displayed on a PC screen for real time monitoring (Figure 3.8). Real time monitoring aided in reacting quickly to probe cut outs, faulty connections, and abnormal behavior in the reactor (e.g. low buffering



capacity) and in monitoring the extent of algal growth. Algal growth can be displayed in terms of pH and DO changes. Due to the addition of  $\text{CO}_2$ , the pH decreases instantly. Algal growth can be determined by monitoring the increase in pH between  $\text{CO}_2$  additions, in particular the amount and steepness of increase (see Section 2.7 & 4.5). Likewise the concentration of dissolved oxygen can give an indication of algal growth. When algae grow, they produce oxygen during the light period and reduce some of the oxygen due to respiration during the dark period (see Section 2.7).

After the experiment concluded, the data was processed in Microsoft Excel. This included adjusting errors in the time logging and substituting zero readings with averages of previous and proceeding readings where the DO or pH meter showed short cut-outs in the data set. In addition the time data had to be translated into time elapsed and adjusted to Matlab data formats. Further additions include indications of day and light periods. Matlab was used to read the Excel spreadsheets and display the data in charts. Additional information regarding data interpretation is provided in Section 3.6.

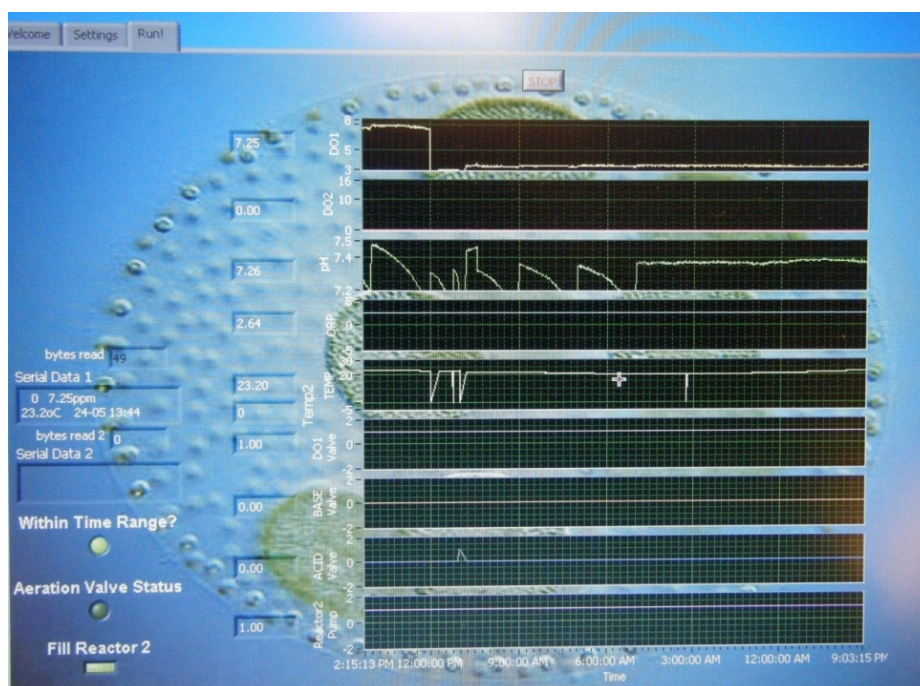


FIGURE 3.8 THE LABVIEW SOFTWARE PACKAGE LOGGS DO, pH, ACID AND BASE ADDITION DATA AS WELL AS TIME.

### 3.5.4 MICROALGAE HARVEST

The algae were harvested after the death phase had occurred. Centrifugation was used to separate the algae from the wastewater. The centrifugation occurred at 8000 rpm for 10 minutes with a 3 minute cool-down (Beckman Avanti Centrifuge J-25 I). The algae were washed once with distilled water and then underwent centrifugation again at 4000 rpm for 10 minutes (Eppendorf Centrifuge 5810 R). The algae pellets were frozen in a freezer for 24 hours and then freeze dried (VirTis 2KBTES-55) (Figure 3.9) at  $-56^{\circ}\text{C}$  and 30 torr and stored in a desiccator at room temperature for a later lipid measurement.



FIGURE 3.9 FREEZE DRYER (VirTis 2KBTES-55)

### 3.5.5 DETERMINATION OF ALGAL DRY WEIGHT

Petri dishes were weighted before algae were added. Following centrifugation, the algae were poured into the pre-weighted petri dishes. After the algae were freeze dried, the dish with algae was weighted again. A subtraction of the two weights gave the algal dry weight as shown in Equation 3.8.

$$(petri\ dish + algae) - petri\ dish = algae \quad (Eqn. 3.8)$$

### 3.5.6 DETERMINATION OF TOTAL LIPID CONTENT

To determine the algae's total lipid content, the Folch method (Folch et al 1957) was used. The following steps were taken:

The freeze dried algal cells were first homogenised with chloroform and methanol. The final volume in millilitres of chloroform and methanol was 20 times the algal weight in grams. The ratio of methanol to chloroform was 1:2. 1/5<sup>th</sup> of the total solvent volume was added to the mix as water. A 20 minutes agitation (shaking) of the mix followed. Shaking occurred at 25°C and 150 rpm. The mix was then filtered through fluted filter paper to recover only the liquid phase. The test tube could be rinsed with an additional 1.5 ml methanol to recover more liquid phase if necessary. Then 1/5<sup>th</sup> of the total volume was added as water to the flask. The mix was then placed into a vortex for 10 seconds to allow full mixing. To separate the liquid and the chloroform phase, the mix was then centrifuged at 2000 rpm for 5 minutes. The upper phase/non-chloroform phase was then siphoned off. The chloroform phase, which contains the lipids, was poured into pre-weighted flasks and dried under a nitrogen stream (Figure 3.10). The flasks containing the lipids were then weighted again. The total lipid weight was determined as shown in Equation 3.9. The total lipid percentage was calculated as shown in Equation 3.10.

$$\begin{aligned} & \text{total lipid weight (g)} \\ &= \text{weight of flask and dried lipids} - \text{weight of flask} \quad (\text{Eqn. 3.9}) \end{aligned}$$

$$\text{total lipid content(\%)} = \frac{100}{\text{algal dry weight}} \text{total lipid weight} \quad (\text{Eqn. 3.10})$$

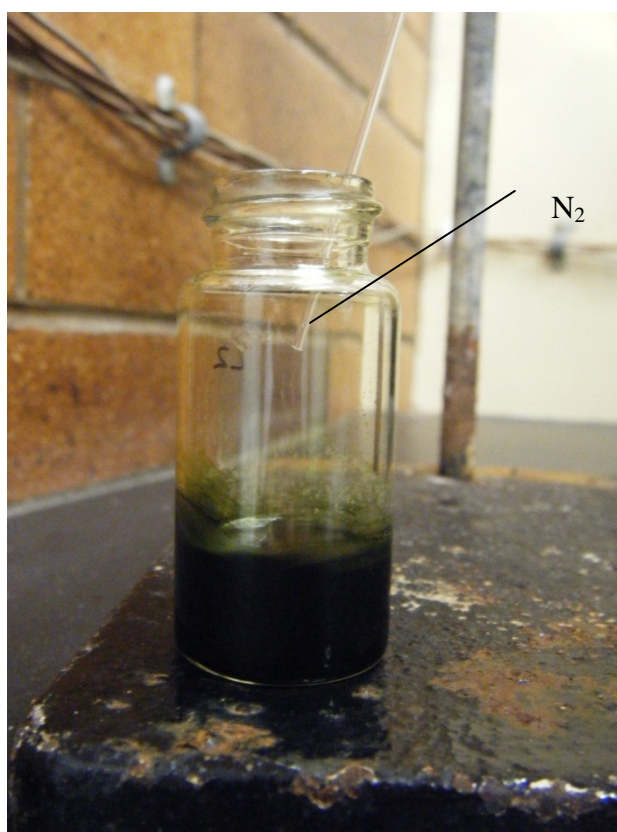


FIGURE 3.10 A NITROGEN STREAM WAS USED TO DRY THE LIPIDS

### 3.5.7 POLAR AND NEUTRAL LIPIDS SEPARATION

Neutral and polar lipids were separated using column chromatography. A vertical glass column was filled with 70-325 mesh ASTM Kieselgel 0.05-0.2 mm (silica gel) at a height of 20 cm (Figure 3.11). The column was then wetted with  $\text{CHCl}_3$ . The dried lipids from the total lipid content determination (Section 3.5.6) were suspended again with chloroform. The re-suspended lipids were then added to the column. Approximately 80 ml of additional chloroform was then added to the column. A beaker below the column caught the neutral



lipids. The polar lipids remained in the column held by the silica gel. The neutral lipid-chloroform mix was then distilled in a Florence flask until mainly lipids remain (Figure 3.12). The neutral lipids were then poured into pre-weighted flasks and dried under a nitrogen stream. After drying the flask with neutral lipids were weighted again. The percentage of neutral lipids was calculated similar to Equation 3.10.

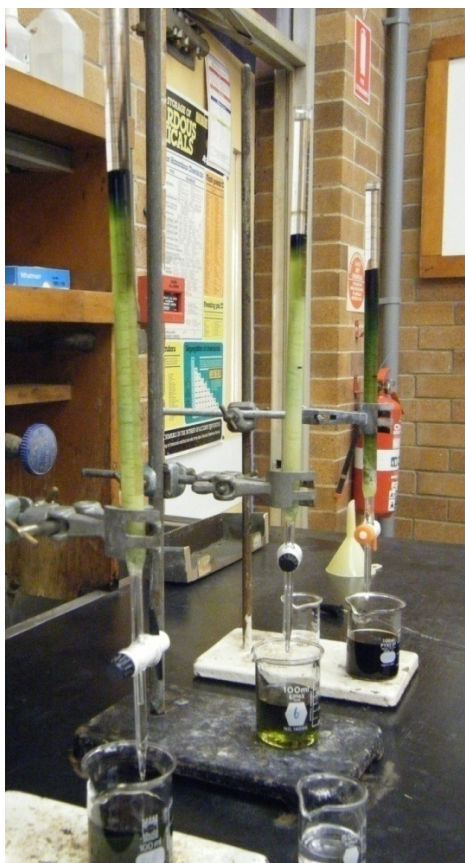


FIGURE 3.11 COLUMN CHROMATOGRAPHY WAS PERFORMED TO SEPARATE NEUTRAL AND POLAR LIPIDS



FIGURE 3.12 THE NEUTRAL LIPID-CHLOROFORM MIX IS DISTILLED IN A FLORENCE FLASK TO SPEED UP THE DRYING PROCESS OF THE LIPIDS

### 3.5.8 TRANSESTERIFICATION

Transesterification was carried out in order to obtain fatty acid methyl ester (FAME) for the bio-diesel production. The following describes a modified transesterification method for the FAME extraction by Larsen & Harris (pers. comm. Kim Larsen & Peter Harris, August 2010). The original method was developed by Ehimen et al (2010).

The algal biomass samples used in this experiment were <1 g. The biomass was combined with 1.5 ml methanol and 55 µL 0.04 M sulfuric acid in a screw cap vessel and heated at 60°C for 4 hours. Stirring occurred for the first hour. After the transesterification the mixture was allowed to stand for one hour to settle the content in the vessel. The reaction mixture was filtered and then washed twice by re-suspension with 0.75 ml methanol for 10 min. To separate hydrophilic components of the extract, 1.25 ml water was added to the filtrate. Then 0.75 ml hexane was added. The hexane layer was later siphoned off and the remaining mix was evaporated to obtain FAME yield. The composition of the FAME was measured using a gas chromatograph/mass spectroscopy (Shimadzu GCMC-QP2010 Plus).

### 3.6 DATA ANALYSIS

#### 3.6.1 NUTRIENT UTILISATION CALCULATIONS

As evaporation of the wastewater significantly altered nutrient concentrations, evaporation had to be accounted for in the nutrient depletion measurements. In order to be able to take evaporation into account, the water level and hence the water volume was measured on a daily basis. Then the total water extraction for the daily 20 ml samples to date was subtracted from the current water volume. The measured nutrient concentration in mg/L, the adjusted water volume in litres and the time elapsed in days was entered into an Excel spreadsheet. The volume and nutrient concentration were multiplied to result a nutrient reading in mg. Then the unconsumed nutrients were calculated. This was done as shown in Equation 3.11.

$$\text{unconsumed nutrients } \left( \frac{\text{mg}}{\text{L}} \right) = \frac{\text{initial nutrients (mg)}}{\text{current water volume (L)}} \quad (\text{Eqn. 3.11})$$

The initial nutrient concentration in mg/L was adopted for the initial unconsumed nutrient. As the initial measurement was vital for the accuracy of evaporation adjusted nutrient data, each initial measurement was taken twice and averaged. For the evaporation adjusted nutrient data, the current nutrient concentration in mg/L was multiplied by the initial unconsumed nutrient concentration in mg/L and divided by the current unconsumed nutrient concentration as demonstrated in Equation 3.12.

$$\begin{aligned}
& \frac{mg}{L} \text{ evaporation adjusted nutrient concentration} \\
& = \text{nutrient concentration} \left( \frac{mg}{L} \right) \\
& * \frac{\text{average initial unconsumed nutrient concentration} \left( \frac{mg}{L} \right)}{\text{unconsumed nutrient concentration} \left( \frac{mg}{L} \right)} \quad (\text{Eqn. 3.12})
\end{aligned}$$

This adjustment was done for all nutrient and growth measurements including suspended solids and optical density.

### 3.6.2 SPECIFIC GROWTH RATE CALCULATIONS

The specific growth rate was calculated for nutrient depletion and oxygen release. For the first case, the slope of the nutrient change over a time period was divided by the slope of the suspended solids over a time period as shown in Equation 3.13. For the oxygen production during the day time, the area under the dissolved oxygen graph over 24 hours was multiplied by the volume of wastewater in the bio-reactor and divided by the light period time as shown in Equation 3.14. The DO values during the dark period were considered to form the baseline, where the increase above the baseline was considered the DO production by the algae which resembled the area under the DO graph.

$$\text{specific growth rate} = \frac{\text{slope} \left( \frac{\text{nutrient}}{\text{time}} \right)}{\text{slope} \left( \frac{\text{suspended solids}}{\text{time}} \right)} \quad (\text{Eqn. 3.13})$$

$$\begin{aligned}
& mg \text{ oxygen produced during daytime} \\
& = \frac{\text{area under DO graph}}{\text{light period}} \text{ wastewater volume} \quad (\text{Eqn. 3.14})
\end{aligned}$$

### 3.6.3 ONLINE DATA MANIPULATION

LabView automatically entered all online data into an Excel spreadsheet. This data was imported into a new spreadsheet. The dissolved oxygen readings were adjusted to 20°C equivalents through the use of automated lookup tables and temperature data collected by LabView. The time collection by Labview was in form of text (serial number) in hours, minutes and seconds e.g. 9.30 pm was shown as 213000. Since the data would later be read into Matlab, the time readings had to be changed to decimal days. In order to do this, the LabView time was split from text to columns and dates were entered. This allowed a precise calculation of the time duration between readings. As the LabView software had to be stopped every few days for maintenance or probe calibrations, the time between measurements was not always the same. It followed that the durations could be added up to produce time elapsed in hours and finally in days e.g. 5:23:01:39 for 5 days, 23 hours, one minute and 39 seconds. Excel was then used to translate this number into decimal days e.g. 5.9594792. A Matlab function file was used to read the Excel spreadsheet and create graphs. Due to the large size of data, Excel could not be used to display graphs and therefore Matlab was used to produce graphs.

After DO and pH graphs were produced using Matlab, and algal growth graphs (suspended solids and optical density) were produced using Excel, it was possible to identify different algal growth phases. Based on this information, time elapsed was re-calculated for selected 24-hour spans that produced pH and DO behaviour for different growth phases over one day.

Based on the time, a 3 for light period and a 1 for dark period was entered into an additional column in the Excel spreadsheet. It was possible to present this data set in the DO and pH graphs to gain an understanding of light and dark periods within the graphs.

Errors in the DO and pH readings, as well as errors in the time readings were adjusted manually. Missing DO and pH data was filled in through linear approximation. The time readings had incorrect entries between 11 pm and 1 am due to a programming error in the LabView software.

## 3.7 RISK MINIMISATION

### 3.7.1 CLOGGING OF DRAINS DUE TO ALGAE GROWTH

If media containing *Chlorella vulgaris* is discharged into drains, there is a high risk of algae growth in the pipe works resulting in clogging of drains. Whilst small amounts (e.g. residue from optical density measurements) were discharged in the laboratory's sinks, large amounts were autoclaved and then properly discharged.

### 3.7.2 BACTERIAL MANAGEMENT

As wastewaters may contain a vast amount of bacteria (even after microfiltration) which pose a risk of bacterial contamination, the spread of bacteria had to be minimised. This was done through the use of 70 % ethanol as a cleaning agent and autoclaving the wastewater medium after use.

### 3.7.3 DISEASE PREVENTION

When handling municipal wastewater, there is a risk of contracting Hepatitis A and B. A vaccination was required prior to handling such wastewaters. In addition, the use of gloves, protective glasses and lab coat was required.

## 3.8 CHAPTER SUMMARY

This chapter discussed the collection and preparation of wastewater, methods of determining the wastewater characteristics as well as calculation methods used for mixing wastewaters from different sources and possible nutrient additions. The set-up of the bio-reactor was described. An overview was given for monitoring and logging pH, DO and temperature changes within the bio-reactor as well as methods of sampling for the determination of algal growth and nutrient depletion. This included data preparation in Microsoft Excel and Matlab. Methods for microalgae harvesting, freeze drying and lipid extraction were explained.

## **CHAPTER 4            NUTRIENT REMOVAL AND LIPID PRODUCTION OF *CHLORELLA VULGARIS* MICROALGAE GROWN IN NURSERY RUNOFF WASTEWATER**

This chapter provides the results and discussions on nutrient removal, growth behaviour, fluctuations of pH and dissolved oxygen and lipid production of *Chlorella vulgaris* in leafy vegetable nursery runoff wastewater. There were two batch experiments performed on this wastewater. The first experiment provided some indication of the general performance of the algae in the nursery wastewater, whilst the second batch experiment served for in-depth data analysis.

### **4.1    BATCH EXPERIMENT 1 (PRELIMINARY EXPERIMENT)**

For the nursery runoff water, a preliminary test was performed. This test was designed to identify shortcomings and problems with the nursery runoff batch experiment. In the preliminary test the wastewater had a N:P ratio of 22:5 and a 5-day BOD of 160 mg/L. The water was a mix of eight parts runoff water, one part fertilizer tank water and factory water each. The water was stored at 4°C for 19 days. Nitrogen was removed, but phosphorous remained largely unchanged.

The suspended solids and spectroscopic measurements produced similar results; hence they both could be used as a growth indicator in future experiments. However, future improvements could be made in the suspended solids measurements in terms of taking a uniform amount of sample, such as 20 ml. This was not done for the first batch experiment, which means standard methods were initially not followed. Figure 4.3 depicts a graph of optical density and suspended solids measurements.

The DO data showed some gaps as the connection between the DO meter and the PC cut out frequently during the night (dark period). This may have been due to a faulty USB serial adapter. It was also found that oxygen bubbles frequently attached to the DO meter causing an artificial increase of DO readings (see Figure 4.1). In later experiments the probe was installed in a different location within the reactor and underwent frequent shaking to remove the bubbles.

The CO<sub>2</sub> was initially designed to be fed every 2 hours from the first day of *Chlorella vulgaris* inoculation. However, it was found that the buffering capacity of the wastewater was too low, which resulted in significant pH fluctuation and a large volume of acid and base addition. As a result, the 2-hour CO<sub>2</sub> addition was discontinued until the algae were adapted to the new condition and started growing (here after 5.5 days). The algae died between 10 and 12 days after inoculation. As nutrients (N and P) were still present, the algae did not run out of N and P supply, but reduced micronutrients may have contributed to the early death. The 5-day BOD was also relatively high and could have promoted bacteria growth and bacterial competition. In addition, the water was not used in the bio-reactor until 19 days after collection and filtration. There may have been sufficient time to establish a bacteria colony in the water prior to the experiment. In later tests this time was reduced.

An algal dry mass of 0.1 g, with 25.47 % total lipids and 7.57 % neutral lipids was determined.

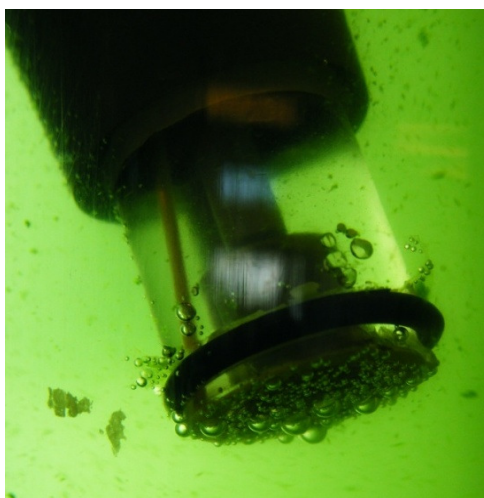


FIGURE 4.1 OXYGEN BUBBLES ON THE DO PROBE.

## 4.2 BATCH EXPERIMENT 2 - RAW WASTEWATER CHARACTERISTICS

Wastewater for the second nursery batch was collected from the nursery runoff water, the fertiliser tank of the nursery and from the drain of the lettuce factory. The characteristics of the individual wastewaters are shown in Table 4.1. After analysing several ratios of the wastewaters for the ultimate algae media, it was found that a pure media of only nursery runoff water gave the most suitable conditions in terms of nutrients and organic strength. The wastewater characteristics used for algal growth in the second experiment are shown in Table 4.2. The runoff water used had a 5-day BOD of 113 mg/L, with nutrient concentrations of 116 mg TN/L, 0.508 mg NO<sub>2</sub>-N/L, 41.1 mg NO<sub>3</sub>-N/L and 23 mg PO<sub>4</sub>-P/L, which resulted in a TN:P ratio of 51:10.

TABLE 4.1 WASTEWATER CHARACTERISTICS OBTAINED FROM THE NURSERY RUNOFF, FERTILIZER TANK AND LETTUCE FACTORY

Mixes			
	Runoff	Fertilizer	Factory
TN (mg/L)	116.25	234.05	31.6
P (mg/L)	22.715	53.368	5.919
TOC (mg/L)	-	-	-
BOD (mg/L)	112.6	20.9	-

TABLE 4.2 SELECTED WASTEWATER MIX WITH FINAL NUTRIENT VALUES AND ORGANIC STRENGTHS

	Runoff	Fertilizer	Factory		
	Parts				
	10	0	0		
	Amounts			Total	N:P Ratio
TN (mg/L)	116.25	0	0	116.25	5.1
P (mg/L)	22.715	0	0	22.715	1
TOC (mg/L)	-	-	-	-	-



### 4.3 GROWTH CHARACTERISTICS

Figure 4.2 shows the growth characteristics of the algae in terms of optical density and suspended solids. The lag phase could be identified until approximately day 4. The exponential growth phase occurred between day 4 and 6; followed by a linear growth trend until day 12. The stationary phase remained until approximately day 15, followed by the death phase.

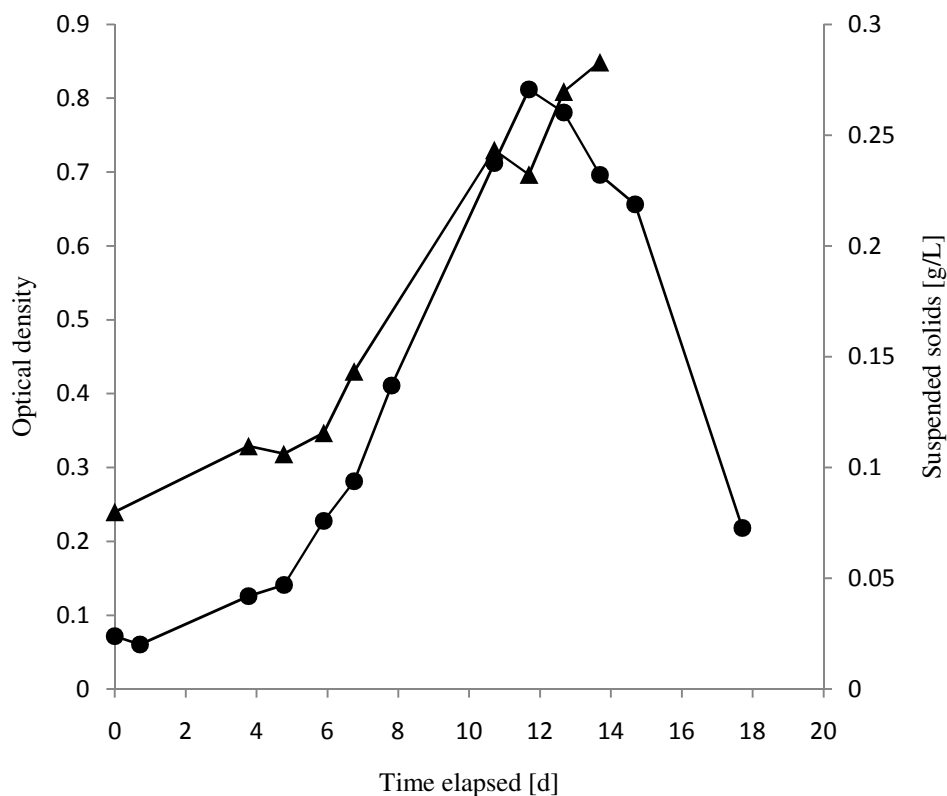


FIGURE 4.2 ALGAL GROWTH IN NURSERY WASTEWATER WAS MONITORED BY OBSERVING DAILY CHANGES BY MEASURING THE ABSORBANCE AT THE OPTICAL DENSITY (●) AT 505 NM WITH A SPECTROPHOTOMETER AND SUSPENDED SOLIDS (▲)

Figure 4.3 shows a comparison of the growth characteristics between the first and second nursery batch experiments. The first experiment performed poorly in terms of algal growth compared to the results of the second experiment. This may have been due to the reasons

outlined in Section 4.1. In addition, there was also significantly less suspended solids data available for the first experiment, due to limited laboratory access during the first experiment.

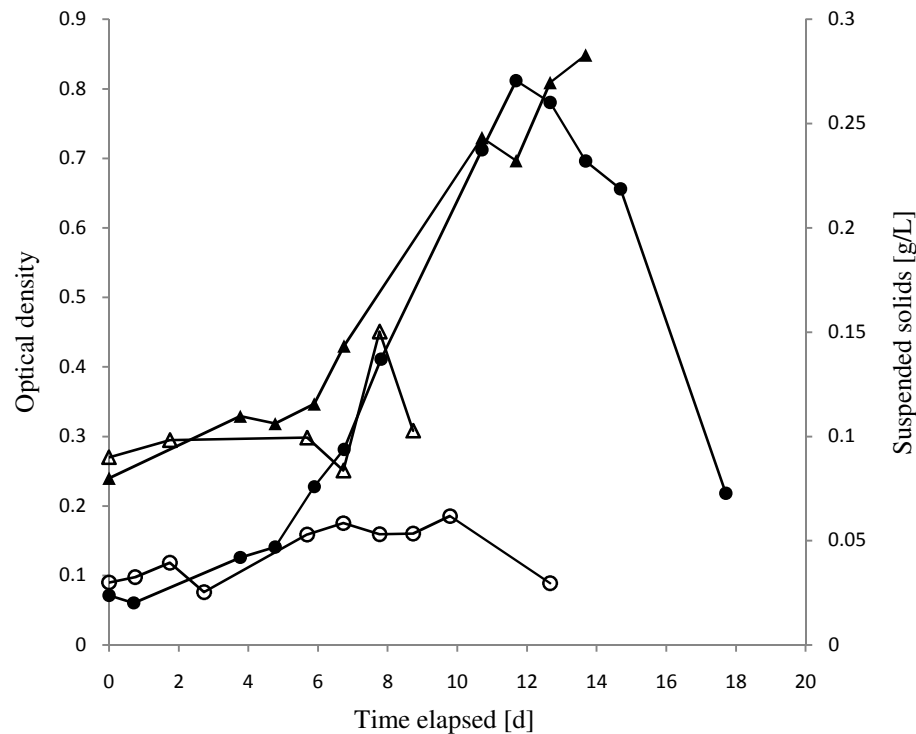


FIGURE 4.3 COMPARISON OF ALGAL GROWTH BETWEEN THE FIRST AND SECOND NURSERY BATCH EXPERIMENT. GROWTH WAS MEASURED WITH OPTICAL DENSITY (○= NURSERY TEST 1, ● = NURSERY TEST 2) AT 505 NM WITH A SPECTROPHOTOMETER AND SUSPENDED SOLIDS (△ = NURSERY TEST 1, ▲= NURSERY TEST 2)

Figure 4.4 shows a relationship between suspended solids and optical density. The relationship between the two can be expressed as shown in Equation 4.1.

$$\text{suspended solids} = 0.2691 \text{ optical density} + 0.0659 \quad (R^2=93.6\%) \quad (\text{Eqn. 4.1})$$

The concentration of suspended solids can be predicted by using the much quicker technique of measuring optical density.

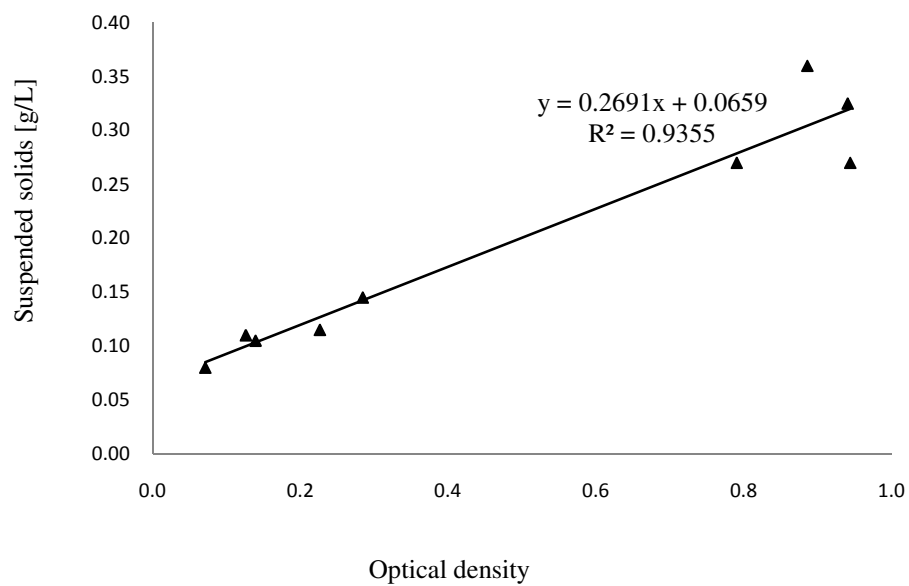


FIGURE 4.4 RELATIONSHIP BETWEEN SUSPENDED SOLIDS MEASUREMENTS AND OPTICAL DENSITY AT 505NM USING NURSERY WASTEWATER.

Figure 4.5 shows filter paper from the suspended solids measurements. It shows the initial discoloration of the algae during the lag phase and the increase in algae mass during the linear phase. The maximum algae mass was achieved in the stationary phase. The death phase again shows discoloration on the filter paper.

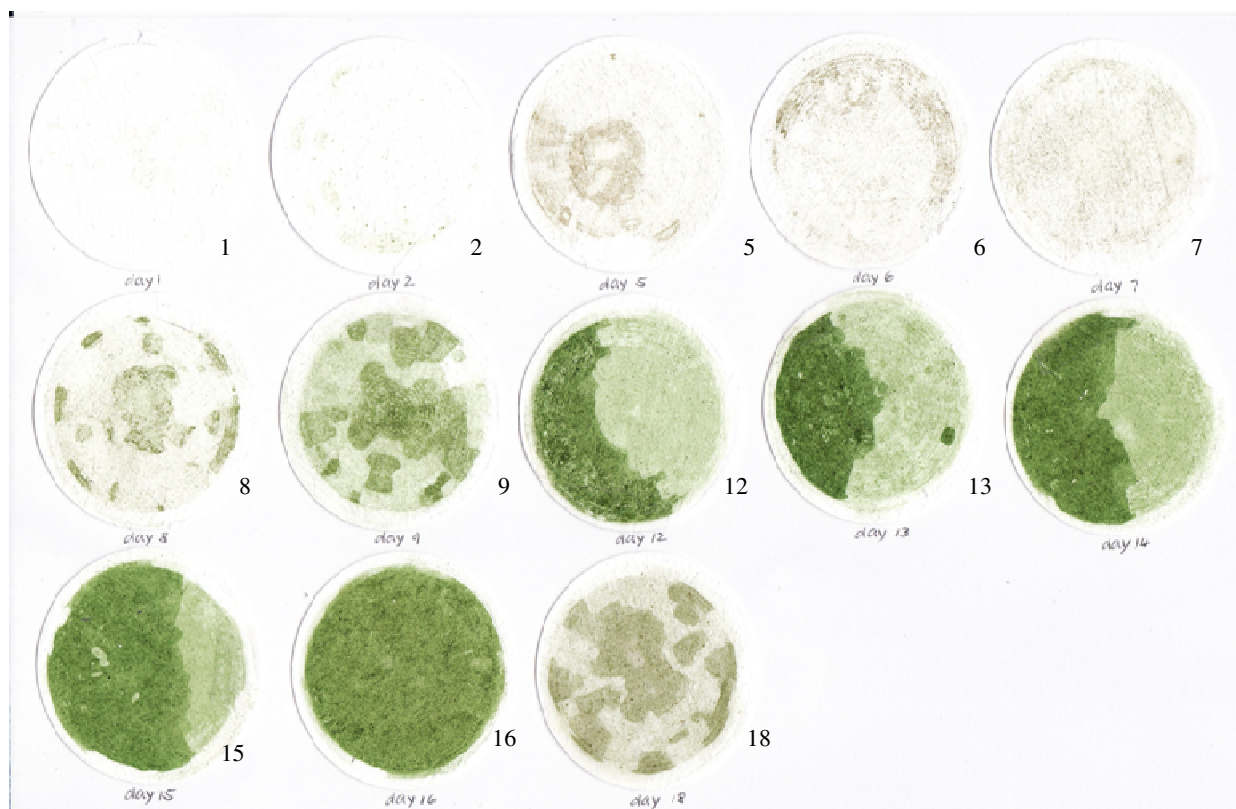


FIGURE 4.5 FILTER PAPER COLLECTED OVER THE GROWTH PERIOD, WHERE THE NUMBERS REPRESENT THE DAY OF FILTRATION.

#### 4.4 NUTRIENT REMOVAL

Figure 4.6 shows the change in nitrogen concentration in the wastewater during the culturing period. Total nitrogen decreased at the rate of 2.64 mg/L/d. The algal growth in terms of suspended solid increment was 28.9 mg SS/L/d. Assuming all the suspended solids were from algal cells, the specific growth rate of algae can be deduced as 0.091 TN/SS. Organic-N and  $\text{NH}_4\text{-N}$  decreased at a rate of 2.04 mg/L/d with a specific growth rate of 0.071 (Org-N +  $\text{NH}_4\text{-N}$ )/SS. Yun et al (1997) found  $\text{NH}_4\text{-N}$  to be depleted at a rate of 20.64 mg/L/d in wastewater of a steel making facility. This can be attributed to the different wastewater characteristics.

Nitrate nitrogen remained unchanged until day 11, after which it decreased at a rate of 1.24 mg/L/d at a specific growth rate of 0.043  $\text{NO}_3\text{-N}$ /SS. It should be noted that the conditions provided were ideal for both autotrophic nitrifying bacteria as well as algal growth. Nitrite

was found to increase from 0.52 to 5.1 mg/L until day 4, which corresponds to decrease of ammonia during the lag phase when there was no algae growth. This could have been due to nitrification where ammonia could be oxidised to nitrite. However, there was no evidence that nitrite nitrogen was oxidised to nitrate nitrogen since the profile of nitrate nitrogen remained the same. After day 12, the nitrite nitrogen increased to 7.51 mg/L again, indicating that when the algae reached its death phase, the nitrifying bacteria may have started oxidising ammonia. However, this cannot be verified unless results are repeated in further experiments.

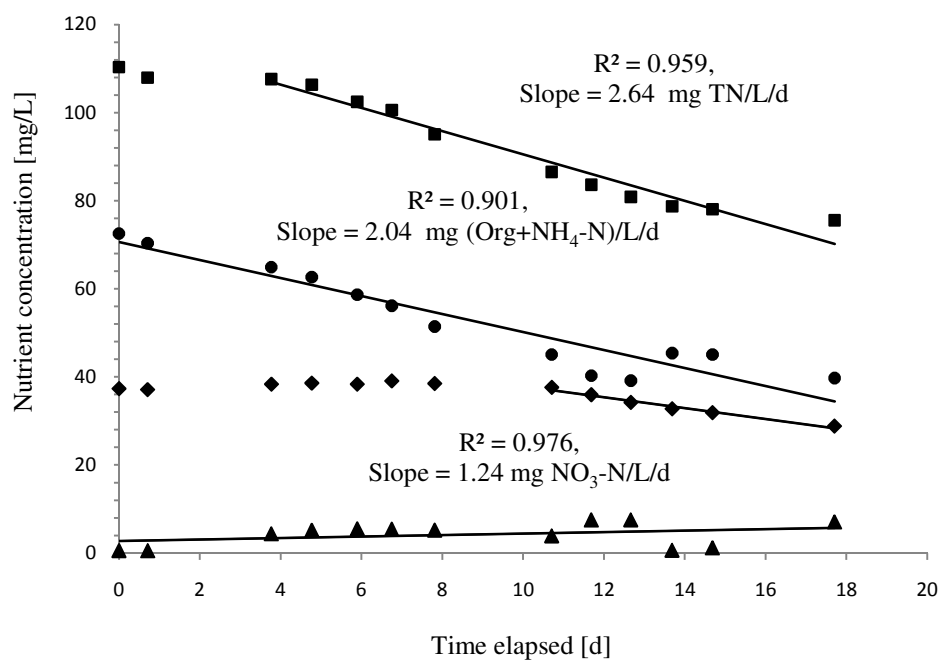


FIGURE 4.6 THE GRAPH SHOWS THE CHANGE IN NITROGEN CONCENTRATION IN FORM OF ORG-N + NH<sub>4</sub>-N (●), TN (■), NO<sub>2</sub>-N (▲), NO<sub>3</sub>-N (◆).

Research has shown that *Chlorella vulgaris* consumes ammonia first before any other form of nitrogen in the wastewater (Grobbelaar 2004, Ruiz-Marin et al 2009, Yun et al 1997, Graham & Wilcox 2000). The same behavior can be observed in this case as shown in Figure 4.6. Also in this experiment, ammonia nitrogen seems to have been preferred nitrogen source until day 11, after which some of nitrate nitrogen appeared to have been taken up.

Phosphate-P depleted at a rate of 0.27 mg/L/d with a specific growth rate of 0.0093 PO<sub>4</sub>-P/SS. Algae require only 1/8<sup>th</sup> -1/16<sup>th</sup> the concentration of P compared to N, hence the slow

consumption rate of P by the algae (Lundquist 2006, Grobbelaar 2004). In these experimental results, TN was used at 2.64 mg/L/d corresponding to a P use of 0.27 mg/L/d resulting in the ratio of 5:49. The depletion of  $\text{PO}_4\text{-P}$  is shown in Figure 4.7.

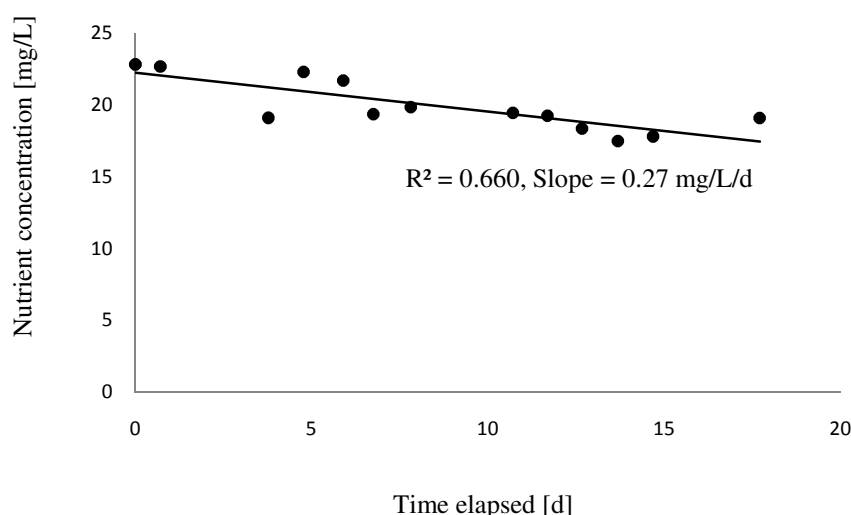


FIGURE 4.7  $\text{PO}_4\text{-P}$  CHANGE WITH TIME.

## 4.5 pH CHANGE WITH TIME

In addition to the continuous 10 ml/min  $\text{CO}_2$  supply,  $\text{CO}_2$  was provided every 2 hours for 15 seconds over 16 hours at a volume of 56 ml/min. The pH rises as the algae consumes  $\text{CO}_2$  and reduces its concentration (Chisti 2007, Fogg & Collyer 1953), because the photosynthetic  $\text{CO}_2$  fixation causes  $\text{OH}^-$  to accumulate in the wastewater (Grobbelaar 2004), while pH decreases as  $\text{CO}_2$  is supplied. Figure 4.8 shows pH dynamics during the 18 day culturing period, in which the pH seems to have steadily increased after day 4.

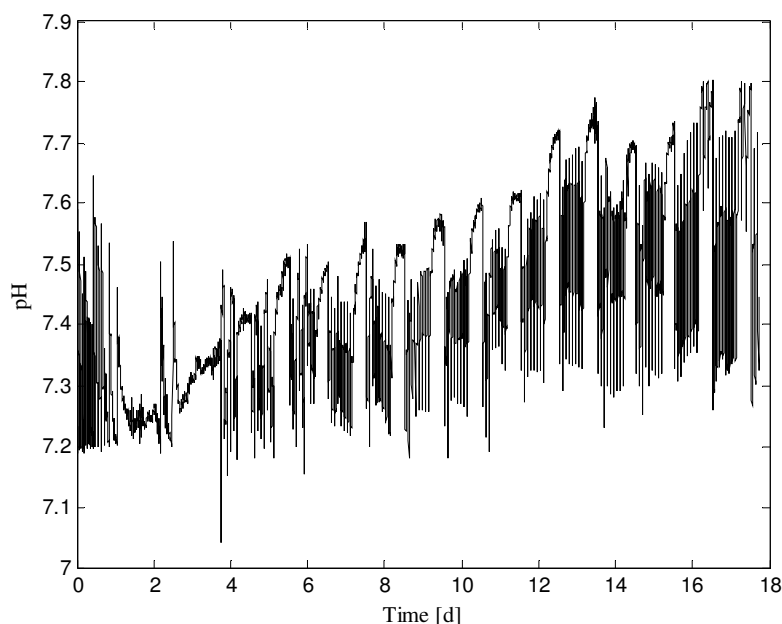


FIGURE 4.8 pH WAS MONITORED EVERY MINUTE FOR 18 DAYS.

Figure 4.9 shows the pH changes that occurred during different growth phases for 24 hours. There was no  $\text{CO}_2$  supplied during the lag phase. Hence, the pH during that phase remained largely unchanged and reached the lower end of the allowable pH range just after 3 hours after commencement of the light period, where it received a sodium hydroxide supply to increase the pH. The unchanged pH may also be due to the consumption of ammonia by nitrifying bacteria, which releases  $\text{H}^+$  and hence decreases pH (Pienkos 2007) and the offset of pH due to  $\text{CO}_2$  consumption by algae from the continuous  $\text{CO}_2$  supply and the atmosphere. In addition, it was possible to observe that no pH change occurred during the 8 hour dark period when the light was off. This is evident in all phases except in the death phase. It is not clear why there was an increase in pH during the death phase, which is contrary to the expected behaviour.

Both the stationary and linear phase demonstrated the algae's growth between  $\text{CO}_2$  supplies. The steepness of the slope indicated utilisation of  $\text{CO}_2$  and hence algal growth. During the linear growth phase, the slope was 0.0945 pH unit/h where during the stationary phase the slope was 0.1428 pH unit/h. This indicates that a larger biomass was available for the consumption of  $\text{CO}_2$  during the stationary phase. For both phases, the slope was slightly shallower for the first two hours until the second  $\text{CO}_2$  addition occurred. This may be due to algae adjusting to the light period after having a rest period and undergoing the dark activity

of photosynthesis. The slope also decreased after the last  $\text{CO}_2$  pumping after the light period has ceased. Algae gains energy from light and without this energy source they consume nutrients at a much slower rate. This confirms findings that the algae stop growing during dark periods and starts growing exponentially as soon as the light period starts again (Widjaja et al 2009).

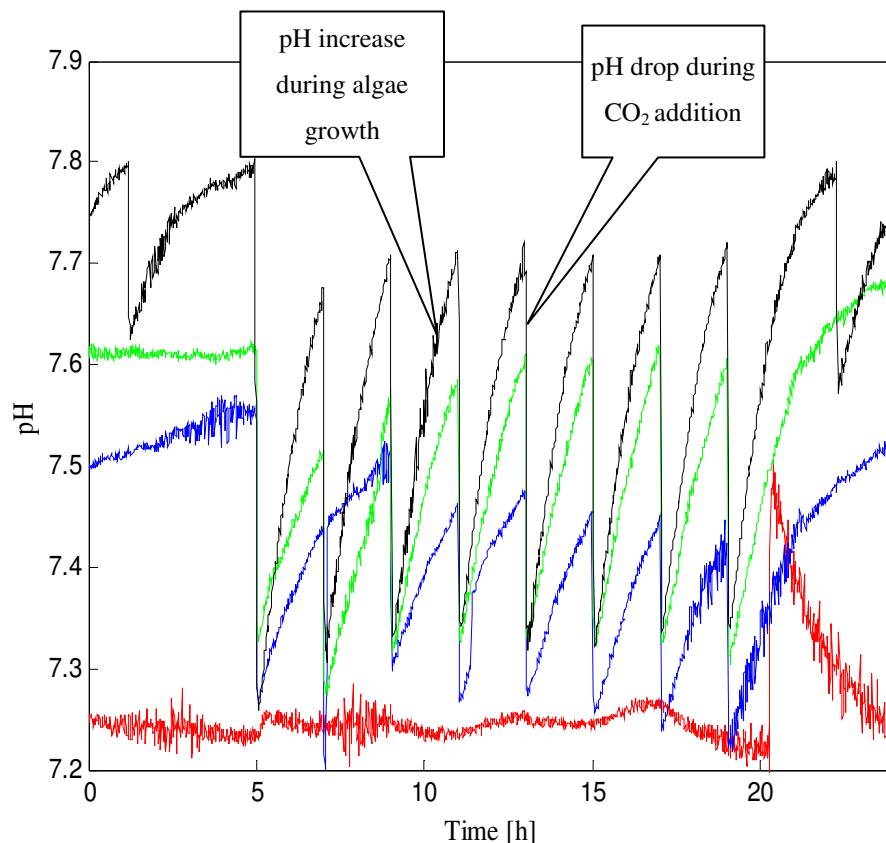


FIGURE 4.9 THE GRAPH SHOWS THE pH INCREASE DUE TO  $\text{CO}_2$  CONSUMPTION BY THE ALGAE DURING THE LIGHT PERIOD AND THE DECREASE OF pH DUE TO  $\text{CO}_2$  ADDITION FOR DIFFERENT GROWTH PHASES: LAG PHASE (—), LINEAR PHASE (—), STATIONARY PHASE (—) AND DEATH PHASE (—).

#### 4.6 DISSOLVED OXYGEN CHANGE WITH TIME

Figure 4.10 shows the concentration of dissolved oxygen for the 18 day culturing period. The bottom graph in Figure 4.10 gives an indication for light (peaks) and dark (valleys) periods. Whilst, during the light period, the algae photosynthesis exceeds the respiration which causes the release of oxygen into the liquid phase, during the dark period there was a



net consumption of oxygen as a consequence of respiration (Masojidek et al 2004). The oxygen production increased linearly until day 10 and then reduced exponentially until day 18 during the death phase.

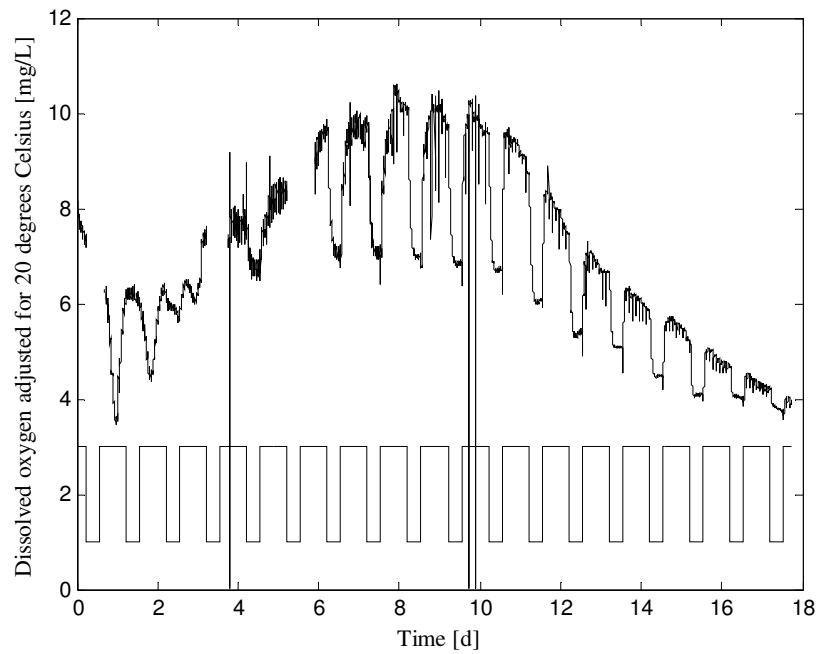


FIGURE 4.10 DISSOLVED OXYGEN WAS ADJUSTED TO 20 DEGREES CELSIUS AND MONITORED EVERY MINUTE FOR 18 DAYS. THE BOTTOM GRAPH INDICATES LIGHT (PEAKS) AND DARK (VALLEYS) PERIODS.

Figure 4.11 shows the average maximum dissolved oxygen reading during the light period and the average minimum dissolved oxygen reading during the dark period. The wastewater was supersaturated for 6 consecutive days during the light period. At 20°C, the saturation value of dissolved oxygen is taken as 9.17 mg/L. Supersaturation occurred during the linear growth phase as indicated by the optical density readings on the same graph, where DO produced exceeded this value. The maximum DO readings during the light period increased and decreased at a much higher rate over the entire growth period compared to the minimum DO readings at night. Figure 4.11 also shows the net oxygen produced during the daytime. Again, the maximum oxygen produced occurred during the linear growth phase.

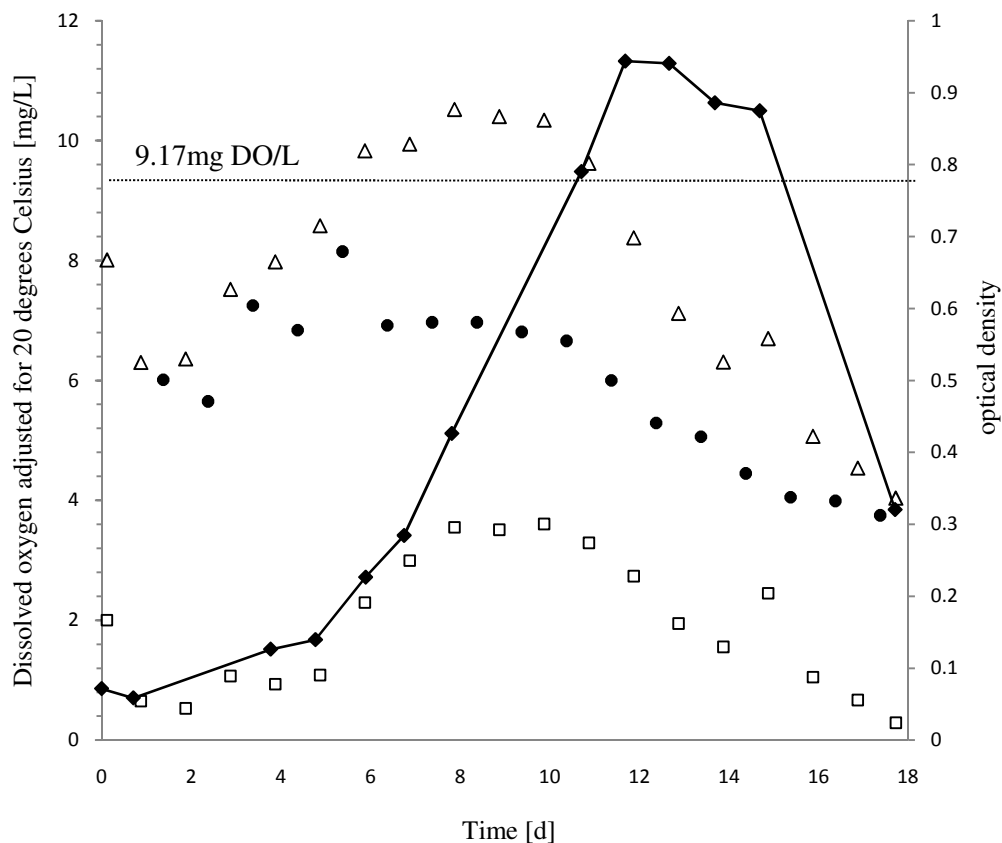


FIGURE 4.11 THE GRAPH SHOWS THE AVERAGE MAXIMUM DISSOLVED OXYGEN READING DURING THE LIGHT PERIOD (Δ), THE AVERAGE MINIMUM DISSOLVED OXYGEN READING DURING THE DARK PERIOD (●), THE NET OXYGEN PRODUCED DURING THE DAYTIME (□) AND THE OPTICAL DENSITY READINGS (◆)

Figure 4.12 shows the concentration of dissolved oxygen for different growth phases over 24 hours. The sudden reduction in oxygen concentration that occurred every two hours during the light period is due to oxygen stripping instigated by CO<sub>2</sub> pumping. It can be seen that there was a much higher oxygen release during the linear growth phase, followed by the stationary phase and death phase, corresponding to net oxygen release rates of 334.9 mg DO, 220.9 mg DO, and 38.2 mg DO respectively. During the dark period, the DO concentration remained between 6 and 7.5 mg DO/L for the linear and stationary growth phase and approximately 4 mg DO/L during the death phase. These values were considered to be the baseline, where the increase above the baseline was considered to be the net DO production by the algae (photosynthesis – respiration).

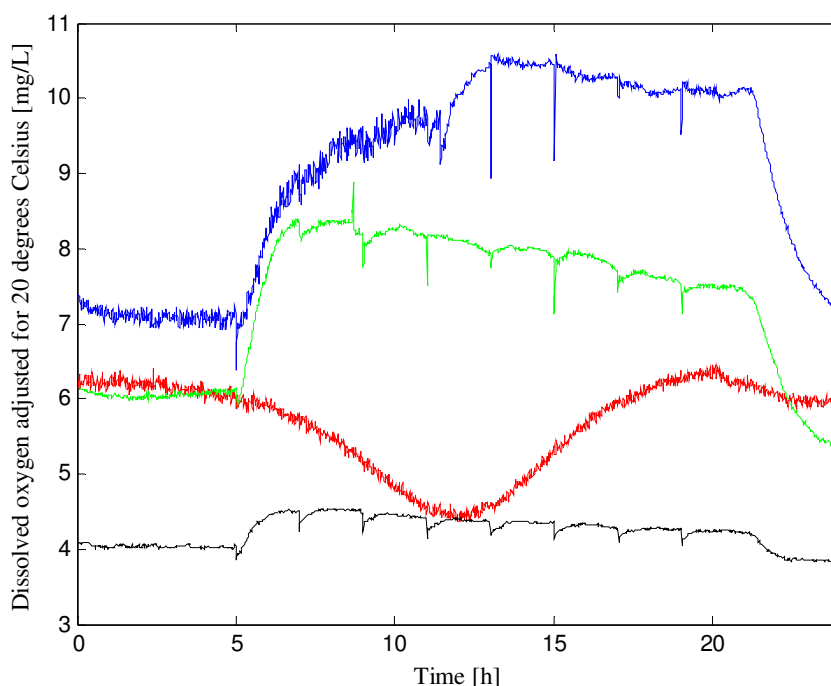


FIGURE 4.12 THE GRAPH SHOWS THE CHANGE IN DISSOLVED OXYGEN CONCENTRATION IN THE BIO-REACTOR FOR DIFFERENT GROWTH PHASES: LAG PHASE (—), LINEAR PHASE (—), STATIONARY PHASE (—) AND DEATH PHASE (—).

#### 4.7 ALGAL DRY MASS AND LIPID CONTENT

The dry mass of the algae (see Figure 4.13) in the second experiment was determined to be 0.67 g, with a total lipid content of 9.66 % and neutral lipids of 4.1 %. In comparison, the algal dry mass for the first experiment was 0.10 g, with 25.47 % total lipids and 7.57 % neutral lipids. The reason for the large reduction in total and neutral lipids in the second batch test was largely due to poor lipid extraction techniques. The algal mass in the second experiment was so large that it was not possible to fit all algal biomass into the vial. It is possible that the remaining mass contained significantly more lipids than the mass that was placed into the vial (pers. comm. K. Larsen, August 2010). For that reason, the lipid content in the second experiment is not representative for nursery wastewater; it is believed to be significantly more.

It is also important to note that the algae were not harvested until the death phase was advanced. Normally the algae would be harvested at the stationary phase where algae mass and lipid content is at its peak. An earlier harvest was not performed because that would not

have provided the whole growth curve, which is important for determining growth characteristics and nutrient removal.

According to Harris (pers. comm. P. Harris, October 2010), the neutral and polar lipid separation was found to be inaccurate. Tests performed by Harris and Larsen found neutral lipids in the polar lipids phase. Therefore it is possible that all neutral lipid data in their research and this research was slightly underestimated. However, it was not possible to determine whether polar lipids were also present in the neutral lipid phase. For that reason it is unclear how accurate the collected data is.



FIGURE 4.13 FREEZE DRIED ALGAL MASS IN A PETRI DISH

#### 4.8 LIMITING FACTORS OF ALGAL GROWTH

As the macronutrients were not fully depleted, it is unlikely that N, P or C were the limiting factors of algae growth and ultimately the cause of algae death. The quality and quantity of light, temperature, oxygen concentration, CO<sub>2</sub>, pH and micronutrients can be possible influencing factors of algal growth (Moheimani 2005). In addition, microorganisms, competition with other algae or shear through vigorous mixing, may contribute to the growth performance of the algae (Moheimani 2005). After light, temperature has the most significant effect of algae growth (Mata et al 2010). While most algae strains can tolerate up to 15°C below their optimum temperature, the growth can be severely inhibited at a temperature rise of 2 to 4°C above the optimum. Mata et al (2010) suggested a constant

temperature of 20 to 26°C, which was achieved for a majority of the time for this experiment. Therefore it is unlikely for increased temperature to be a contributing factor. Supersaturated oxygen in the bioreactor can cause decrease of photorespiration and lead to photooxydative death of the algae. At 20°C, the saturation value of dissolved oxygen is 9.17 mg/L, while the maximum DO reached on day 8 was 9.94 mg/L (Figure 4.11). Elevated oxygen levels have significant effects on algal growth (Moheimani 2005, Richmond 2004). This could be a cause of the early algal death. Another possibility could be the depletion of micronutrients and vitamins. Previous research has shown that micronutrients play an important part in the growth of algae (Moheimani 2005). As there were no micronutrients and vitamins applied with the fertiliser for the nursery seedlings, it is unlikely that all essential micronutrients were available for the algae. Unlike dairy, piggery and municipal wastewater where all the micronutrients are believed to be available for the algae to grow, in the case of nursery wastewater, there is a lack of trace nutrients, thus causing the death of algae in spite of the presence of nutrients. A certain degree of turbulence in the water can result in shearing and death of the algae (Mata et al 2010, Moheimani 2005). In this experiment, turbulence occurs due to mechanical mixing and repetitive aeration during sampling and could therefore have contributed to the algal death. From microscope images it was found that at least one other algae strain (*Euglena*) and some bacteria was present in the wastewater at day 18. It is possible that these organisms competed with *Chlorella vulgaris* for nutrients.

## 4.9 SUMMARY

This batch experiment has given insight into the algal growth rate in the nursery wastewater along with nutrient depletion rate of 2.64 mg TN/L/d and 0.27 mg PO<sub>4</sub>-P/L/d, with the specific growth rate of 0.091 TN/SS. The online measurements including pH and dissolved oxygen can be a real-time indicator of different algal growth patterns. In this experiment, these measurements confirm the algal growth pattern during the whole period, as well as the growth in 24 hours during light and dark periods.

*Chlorella vulgaris* produced 9.66 % total lipids of the algal dry weight and 4.1 % neutral lipids. The first batch experiment also achieved approximately 25.5 % in total lipids and 7.6 % in neutral lipids. It is likely that the values from the second batch experiment were significantly lower than actual lipid contents due to technical errors.

## **CHAPTER 5            NUTRIENT REMOVAL AND LIPID PRODUCTION OF *CHLORELLA VULGARIS* MICROALGAE GROWN IN MUNICIPAL WASTEWATER**

This chapter discusses the experimental results of the nutrient removal, growth behavior, fluctuations of pH and dissolved oxygen and lipid production of *Chlorella vulgaris* microalgae in municipal wastewater, particularly wastewater collected from the inlet of a wastewater treatment plant and a clarifier. There were two batch experiments performed on this wastewater. The first experiment provided some indication of the general performance of the algae in the municipal wastewater, where the second batch experiment served for in-depth data analysis.

### **5.1    BATCH EXPERIMENT 1 (PRELIMINARY EXPERIMENT)**

A preliminary test was performed for the municipal wastewater. This test was designed to identify shortcomings and problems with the municipal wastewater batch experiment. The wastewater had a N:P ratio of 319:5 and a 5-day BOD of 49 mg/L. The water was a mix of five parts (2.5 L) water obtained from the clarifier and two parts (1 L) raw influent. The water was stored at 4°C for 7 days.

During the batch experiment, total nitrogen was removed at the rate of 2.1 mg/L/d with organic nitrogen and ammonia mainly contributing to the nitrogen reduction. Nitrate increased from day 1 to day 2 and then remained largely unchanged. A likely explanation for this is a measurement error on the first day. Phosphorous also increased significantly during the first two days, which was also believed to be incorrect.

The suspended solids showed a growth rate of 5.48 mg SS/L/d. The spectroscopic measurements showed poor correlation to the suspended solids measurements at small optical densities, however, they showed more similar results with the suspended solids at higher

optical densities. A possible reason for that could be poor accuracy of spectroscopy results outside a range of 0.1 to 0.3 (person. comm. P. Harris September 2010).

It was found that the buffering capacity of the wastewater was very low, which resulted in significant pH fluctuation and a large volume of acid and base addition (1 L during the first night). As a result, the 2-hour CO<sub>2</sub> addition and the continuous flow of 10 ml/min were ceased for the first five days.

The algae died between 10 and 12 days after inoculation. The death of the culture was identified through a colour change from green to brown even though the growth measurements could not confirm a reduction in suspended solids or optical density. As nutrients (N and P) were still present, the algae did not run out of N and P supply, but reduced micronutrients may have been contributed to the early death. Due to a possible increased amount of bacterial growth, there might have also been competition between the *Chlorella vulgaris* and bacteria. Furthermore the significant addition of base may have contributed to a substantial increase in sodium. *Chlorella vulgaris* is a freshwater algal strain and prefer low levels of sodium, therefore this may have been a contributing factor of the poor algal growth. The algal dry mass was 0.2218 g, the weight of total lipids was 0.028 g with 12.8 % total lipids based on algal dry weight. Neutral lipids were found to be 5.1% of the algal dry weight. However, the accuracy of the neutral lipid percentage is questionable as stated in Section 4.7.

## 5.2 BATCH EXPERIMENT 2 - RAW WASTEWATER CHARACTERISTICS

The wastewater obtained from the clarifier had the following characteristics as shown in Table 5.1: total nitrogen of 3.18 mg/L, phosphorous of 0.065 mg/L, TOC of 56.54 mg/L and a 5-day BOD of 4 mg/L. The 5-day BOD was obtained from a commercial laboratory due to technical problems at the university wastewater laboratory.

The influent showed total nitrogen of 99.83 mg/L, phosphorous of 5.57 mg/L, TOC of 329.45 mg/L and 5-day BOD of 401 mg/L. This 5-day BOD was also obtained from a commercial laboratory.

Several options for mixing the two wastewater sources were analysed. Table 5.1 shows the selected option with one part of influent (0.5 L) from the wastewater reclamation facility inlet and six parts of effluent (3 L) from the clarifier. The table shows that the initial N:P ratio was 199:10. As this is not an ideal N:P ratio (here P limited), phosphate was added with  $K_2HPO_4$  according to Section 3.2.1. In addition, the amount of nitrogen was also considered to be very low (algae would run out of food quickly). Therefore, not only phosphorous, but also nitrogen was adjusted. Nitrogen was adjusted with  $NaNO_3$  according to Section 3.2.1. The final nutrient concentrations can be found in Table 5.1 'post nutrient adjustment'.

The wastewater mix selected now had a 5-day BOD of 60.7 mg/L and a TOC of 95.5 mg/L. These values showed that there was little organic strength in the mix, which should decrease the possibility of out-competence of the algae due to other microorganisms that prefer a high organic strength. The nutrients in the wastewater had a final concentration of 120 mg TN/L, 0.0 mg  $NO_2$ -N/L, 103.55 mg  $NO_3$ -N/L, 16.63 mg Org.-N+ $NH_4$ -N and 12 mg  $PO_4$ -P/L, which resulted in a TN:P ratio of 10:1.

TABLE 5.1 WASTEWATER CHARACTERISTICS OBTAINED FROM THE CLARIFIER AND INFLUENT

Mixes		
	Clarifier	Influent
TN (mg/L)	3.1675	99.825
P (mg/L)	0.065	5.5705
TOC (mg/L)	56.54	329.45
BOD (mg/L)	4	401



TABLE 5.2      SELECTED WASTEWATER MIX WITH FINAL  
NUTRIENT VALUES AND ORGANIC STRENGTHS

	Clarifier	Influent		
	Parts			
	6	1		
Prior nutrient adjustment	Amounts		Total	N:P Ratio
TN (mg/L)	2.715	14.261	16.976	19.9
P (mg/L)	0.056	0.796	0.852	1
TOC (mg/L)	48.463	47.064	95.527	
BOD (mg/L)	3.429	57.286	60.714	
Post nutrient adjustment	Amounts		Total	N:P Ratio
TN (mg/L)	-	-	120	10
P (mg/L)	-	-	12	1
TOC (mg/L)	48.463	47.064	95.527	
BOD (mg/L)	3.429	57.286	60.714	

### 5.3 GROWTH CHARACTERISTICS

Figure 5.1 shows the growth characteristics of the algae in terms of optical density and suspended solids. The growth phases were not very dominant. A polynomial trend line was fitted and showed maximum growth between day 8 and 10, followed by the death phase. A lag phase could not be identified. A possible reason for such inconclusive results may be the small gain in algal mass over the entire batch experiment. As mentioned earlier, spectrophotometry shows significant error outside of its ideal range of 0.1 to 0.3. Likewise suspended solids measurements would provide incorrect growth patterns at low masses. In addition, there was a possibility that the samples for the optical density and suspended solids measurements were not representative, due to the low algal mass. For example Figure 5.2 shows that some algal mass was attached to the reactor housing and could therefore not be considered in the algal growth measurements. The attached mass was also found to be greener than the settled mass on the reactor's floor.

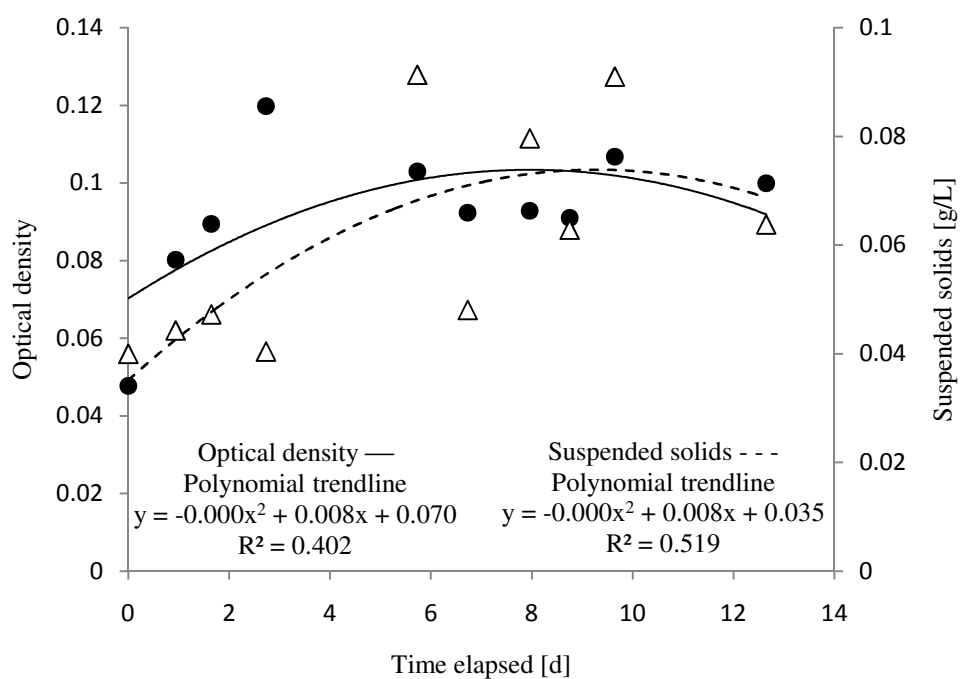


FIGURE 5.1 ALGAL GROWTH IN MUNICIPAL WASTEWATER WAS MONITORED BY OBSERVING DAILY CHANGES BY MEASURING THE ABSORBANCE AT THE OPTICAL DENSITY (●) AT 505 NM WITH A SPECTROPHOTOMETER (JENWAY 6705 UV/VIS.) AND SUSPENDED SOLIDS (Δ)

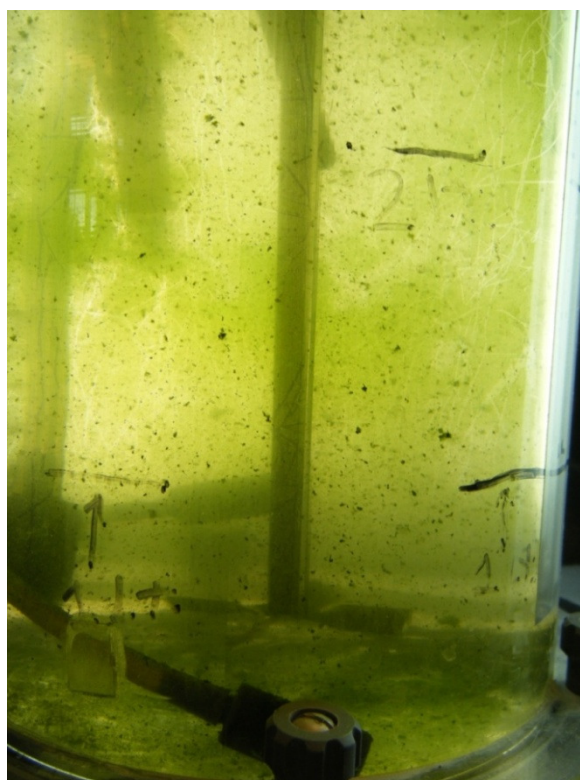


FIGURE 5.2 *CHLORELLA VULGARIS* IN MUNICIPAL WASTEWATER WITH SEDIMENTATION ON THE REACTOR HOUSING AND ON THE FLOOR.

A relationship between suspended solids and optical density can be seen in Figure 5.3. The relationship showed very little correlation, mainly due to the reasons stated above.

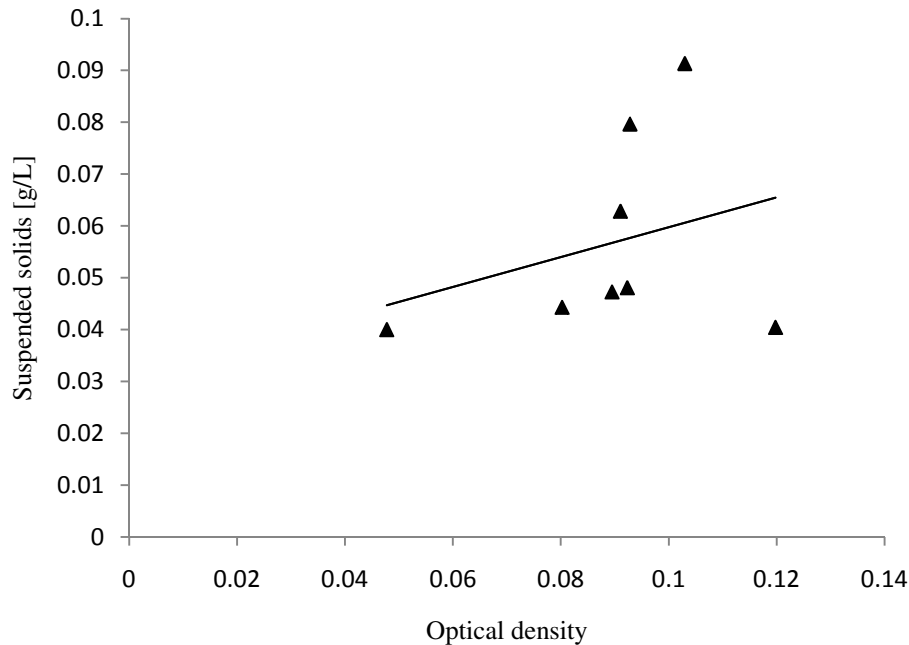


FIGURE 5.3 RELATIONSHIP BETWEEN SUSPENDED SOLIDS MEASUREMENTS AND OPTICAL DENSITY AT 505NM USING MUNICIPAL WASTEWATER.

Figure 5.4 shows filter paper from the suspended solids measurements. It shows a slow increase in algae mass during the linear phase until about day seven. The maximum algae mass (here between day 9 to 11) was achieved in the stationary phase. The death phase showed no prominent discoloration on the filter paper. Generally, the colour of the algal mass was green-brown rather than green.

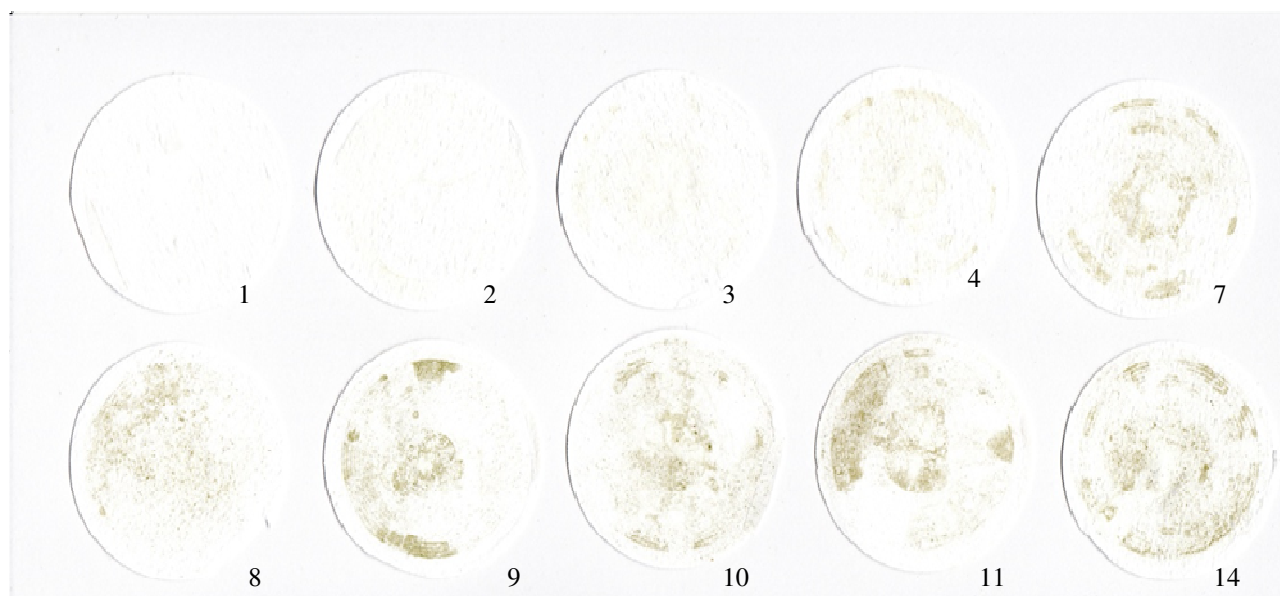


FIGURE 5.4 FILTER PAPER COLLECTED OVER THE GROWTH PERIOD, WHERE THE NUMBERS REPRESENT THE DAY OF FILTRATION.

## 5.4 NUTRIENT REMOVAL

Figure 5.5 shows the change in nitrogen concentration in the wastewater during the culturing period. Total nitrogen (TN) decreased by 1.59 mg/L/d. The algal growth in terms of suspended solid increment was 3.06 mg SS/L/d. Assuming all the suspended solids are from algal cells, the specific growth rate of algae can be deduced as 0.52 TN/SS. Organic-N and  $\text{NH}_4\text{-N}$  decreased at a rate of 1.46 mg/L/d with a specific growth rate of 0.48 (Org-N +  $\text{NH}_4\text{-N}$ )/SS.

Nitrate nitrogen remained largely unchanged until day 5, after which it decreased at a rate of 1.89 mg/L/d at a specific growth rate of 0.62  $\text{NO}_3\text{-N}$ /SS (Figure 5.5). The growth conditions provided are also ideal for autotrophic nitrifying bacteria. Nitrite was found to increase from 1.59 to 2.99 mg/L from day 3 to 12 (Figure 5.6). Even though there was an obvious trend in nitrite increase, bacterial nitrification could not be verified as no attempt was made to identify the bacterial strain.

The organic nitrogen and ammonia data points in Figure 5.5 show that these nitrogen forms were consumed from the first day. Therefore it can be assumed that algae prefer ammonia. But since ammonia was not measured separately, there is a possibility that the algae also consumed organic nitrogen. According to Tsukahara & Sawayama (2005) algae feed on organic and inorganic nitrogen, but they prefer ammonia.

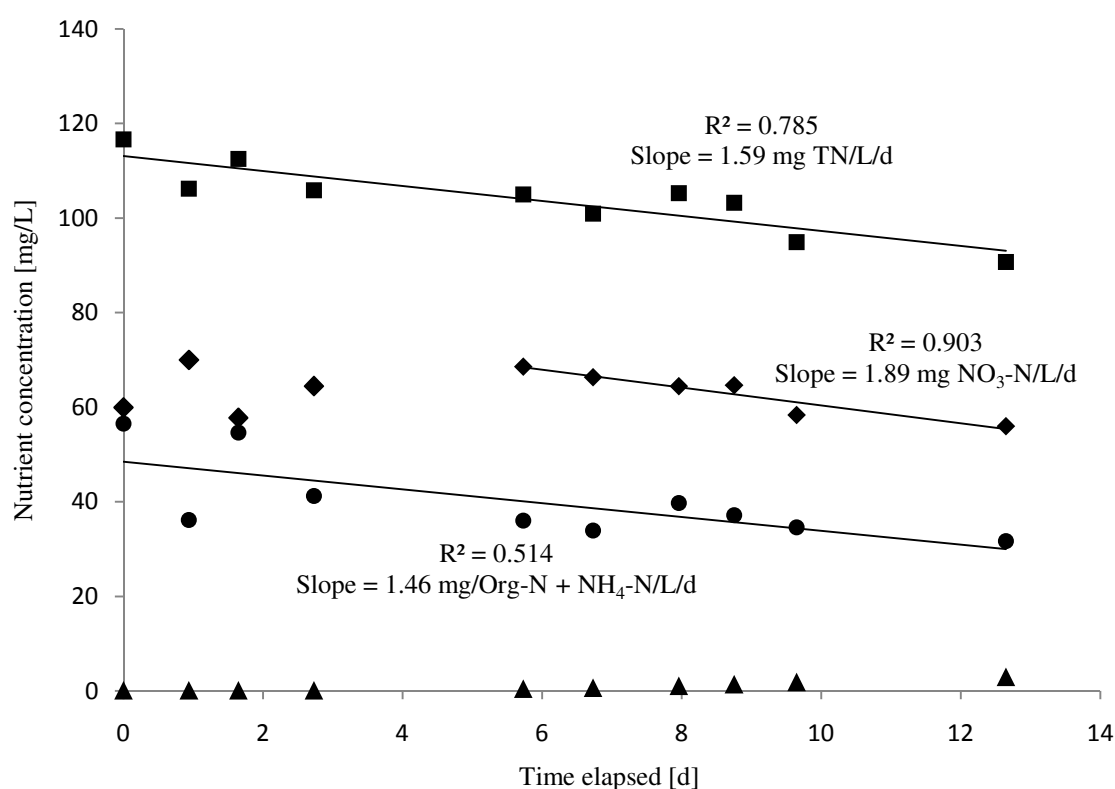


FIGURE 5.5 THE GRAPH SHOWS THE CHANGE IN NITROGEN CONCENTRATION IN FORM OF TN (■), NO<sub>3</sub>-N (◆), Org-N + NH<sub>4</sub>-N (●), NO<sub>2</sub>-N (▲).

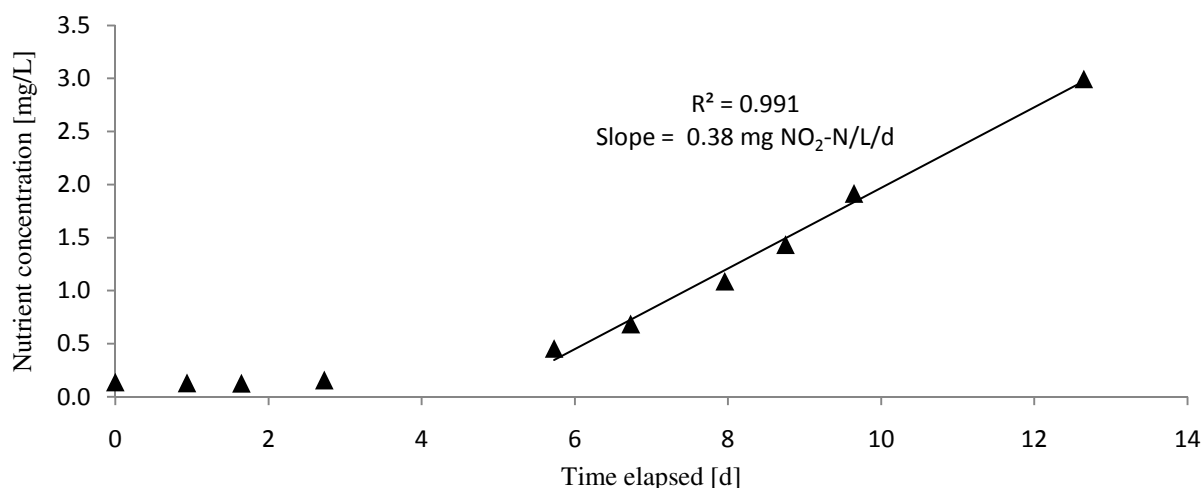


FIGURE 5.6 NITRITE ( $\text{NO}_2\text{-N}$ ) CHANGE WITH TIME

Phosphate-P depleted at a rate of 0.51 mg/L/d with a specific growth rate of 0.17  $\text{PO}_4\text{-P/SS}$ . The depletion of  $\text{PO}_4\text{-P}$  is shown in Figure 5.7. Interestingly TN was used at 1.59 mg/L/d corresponding to a P use of 0.51 mg/L/d resulting in the ratio of 3:1. Algae normally require  $1/8^{\text{th}}$  -  $1/16^{\text{th}}$  of P for every part of N (Lundquist 2006, Grobbelaar 2004). These experimental results showed a much higher utilisation of phosphorous.

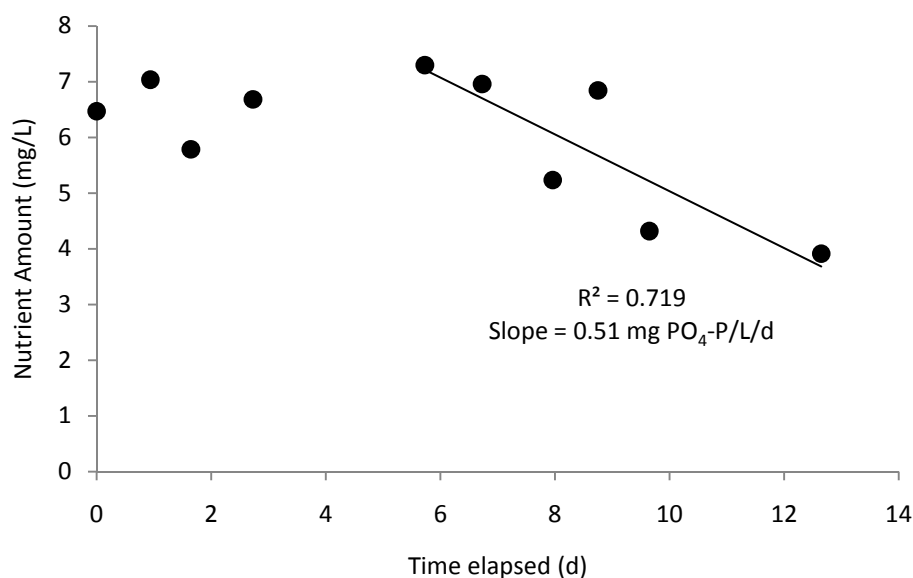


FIGURE 5.7 PHOSPHATE  $\text{PO}_4\text{-P}$  CHANGE WITH TIME.

## 5.5 pH CHANGE WITH TIME

In addition to the continuous 10 ml/min CO<sub>2</sub> supply, CO<sub>2</sub> was provided every two hours for 5 seconds over a 16 hour period at a volume of 56 ml/min. A general trend of pH rise during CO<sub>2</sub> mitigation was evident. Figure 5.8 shows pH dynamics during the 12 day culturing period, in which the pH appeared to be steadily increase after day 3 when the CO<sub>2</sub> feeding was initiated. Base was added to the wastewater shortly after inoculation which resulted in significant pH changes during the first hours. No acid or base was added during a later stage of the experiment.

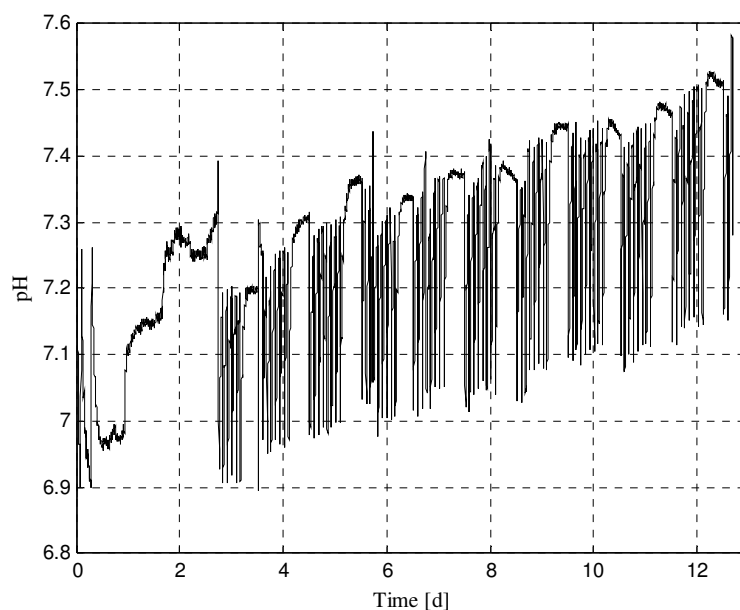


FIGURE 5.8 pH WAS MONITORED EVERY MINUTE FOR 12 DAYS.

Figure 5.9 shows the pH changes that occurred during different growth phases for 24 hours. There was no CO<sub>2</sub> supplied during the first three days as this was believed to be the minimum time frame the algae require to adjust to the new growth medium. Generally the pH in this wastewater appeared to remain below the set point of 7.5. Therefore the pH tolerance range was increased to  $\pm 0.6$ .

It was possible to observe that no pH change occurs during the dark. This is evident in all phases. Both the stationary, linear and the death phase demonstrated the algae's growth between CO<sub>2</sub> supplies. As algal growth should not have taken place during the death phase,

it is believed that the chosen death period was incorrect. It is possible that the death phase had not commenced yet.

The steepness of the pH slope indicates utilisation of CO<sub>2</sub> and hence algal growth. During the linear growth phase, the slope was 0.1115 pH unit/h where during the stationary phase the slope was 0.169 pH unit/h and during the death phase 0.1475 pH unit/h. This indicates that a larger biomass was available for the consumption of CO<sub>2</sub> during the stationary and death phase. For all three phases, the slope is slightly shallower for the first two hours until when the second CO<sub>2</sub> addition occurs. Again, this may be due to *Chlorella vulgaris* slowly adjusting to the light period. The slope also decreased after the last CO<sub>2</sub> pumping after the light period ceased.

Some of the pH data collected may be faulty due to occasional algal settlement on the pH probe especially during the first few days following inoculation. This can be seen in Figure 5.10.



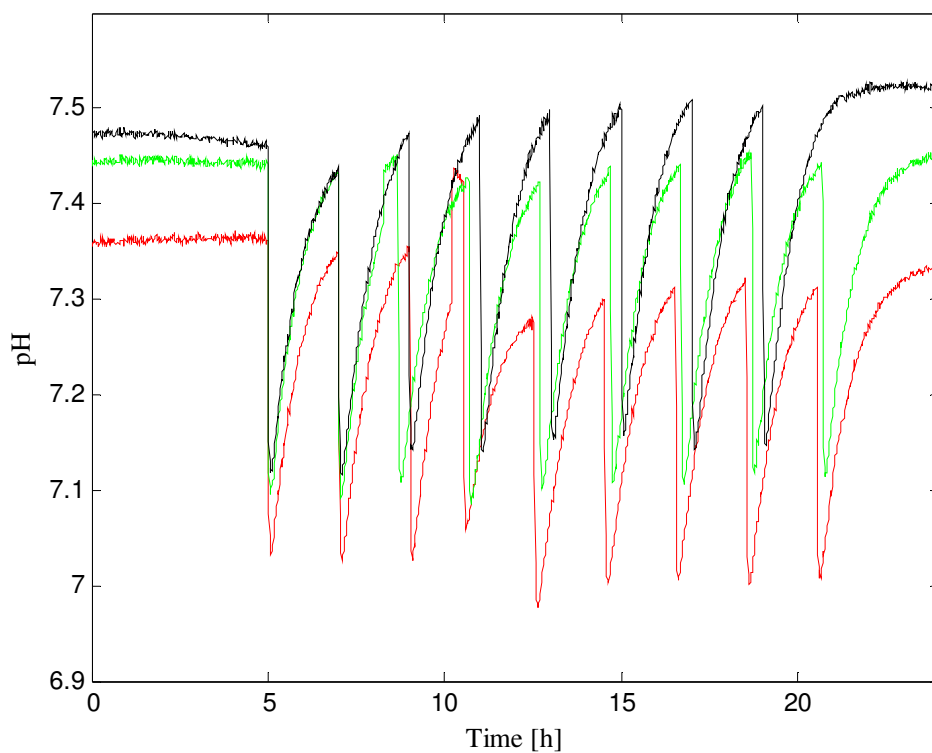


FIGURE 5.9 THE GRAPH SHOWS THE pH INCREASE DUE TO  $\text{CO}_2$  CONSUMPTION BY THE ALGAE DURING THE LIGHT PERIOD AND THE DECREASE OF pH DUE TO  $\text{CO}_2$  ADDITION FOR DIFFERENT GROWTH PHASES: LINEAR PHASE (—), STATIONARY PHASE (—) AND DEATH PHASE (—).

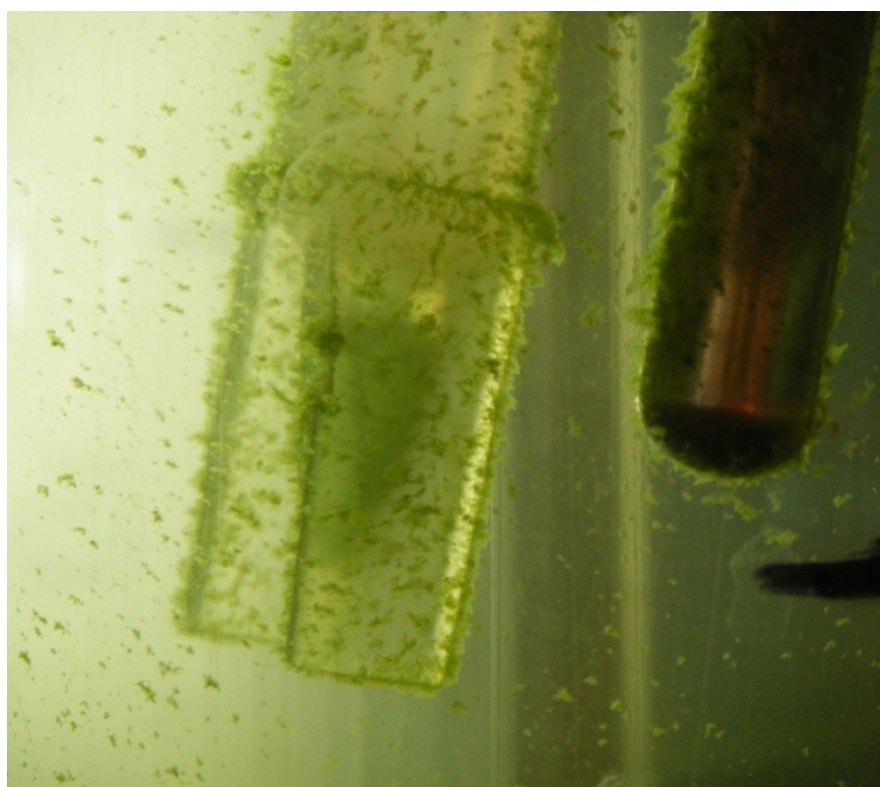


FIGURE 5.10 ALGAL SETTLEMENT ON pH METER

## 5.6 DISSOLVED OXYGEN CHANGE WITH TIME

Figure 5.11 shows the concentration of dissolved oxygen for the 12 days culturing period. The bottom graph in Figure 5.11 gives an indication for light and dark periods. Normally, there is an increase in dissolved oxygen during the light period resulting from photosynthesis and a decrease in dissolved oxygen during the dark period due to the absence of oxygen production and presence of respiration. This typical trend could only be confirmed between day 3 and 7. From day 7 there was an increase of dissolved oxygen at night. This may have been due to a malfunctioning DO probe. Normally, there should also be an increase in oxygen production as algal mass increases. This could also not be confirmed in this batch experiment. Figure 5.12 also shows that net dissolved oxygen did not generally increase from the lag phase to the maximum growth and then decrease again as the death phase was entered.

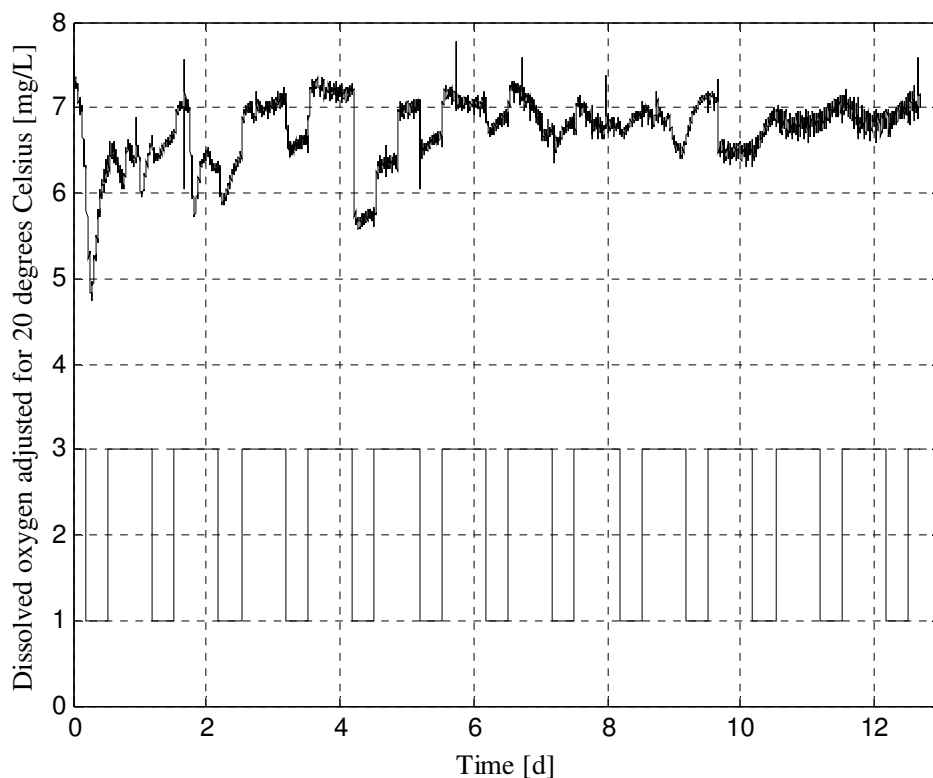


FIGURE 5.11 DISSOLVED OXYGEN WAS ADJUSTED TO 20 DEGREES CELSIUS AND MONITORED EVERY MINUTE FOR 12 DAYS. THE BOTTOM GRAPH INDICATES LIGHT (PEAKS) AND DARK (VALLEYS) PERIODS.

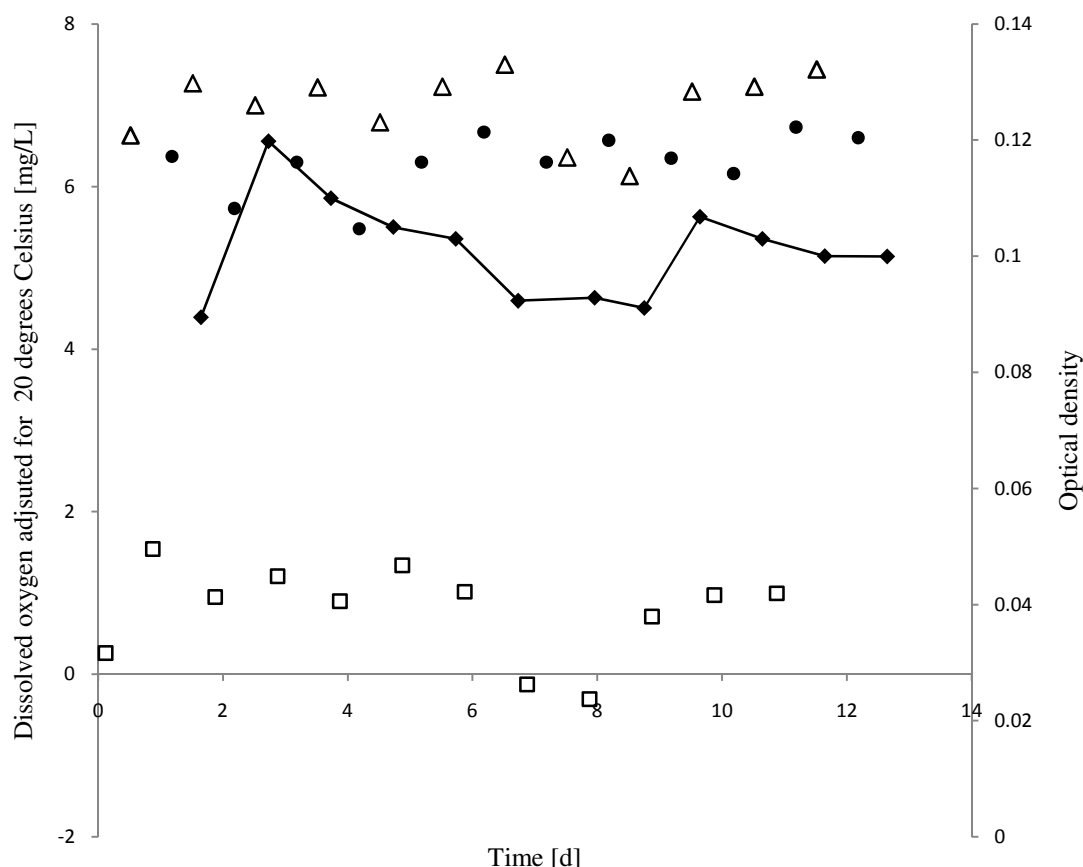


FIGURE 5.12 THE GRAPH SHOWS THE AVERAGE MAXIMUM DISSOLVED OXYGEN READING DURING THE LIGHT PERIOD (Δ), THE AVERAGE MINIMUM DISSOLVED OXYGEN READING DURING THE DARK PERIOD (●), THE NET OXYGEN PRODUCED DURING THE DAYTIME (□) AND THE OPTICAL DENSITY READINGS (◆)

Figure 5.13 shows the concentration of dissolved oxygen for different growth phases over 24 hours. The sudden reduction in oxygen concentration that occurs every two hours during the light period was due to oxygen stripping instigated by CO<sub>2</sub> pumping. The reduction on dissolved oxygen is only minor due to a very short period of CO<sub>2</sub> feeding (only 5 seconds for this batch experiment). It can be seen that there was a slightly higher oxygen release during the linear growth phase, followed by the death phase and stationary phase. Only the linear growth phase showed a typical DO behaviour as dissolved oxygen is higher during the light period and lower during the dark period. The non conformance during the other two growth phases may have been due to oxygen stripping off the DO probe during daily suspended solids sampling. This would mean that oxygen bubbles attached on the DO probe at night time and gave an elevated DO reading until the bubbles were stripped off. The other explanation may be a faulty DO probe.

During the dark period, the DO concentration remained between 6.5 and 6.7 mg DO/L for the linear and stationary growth phase and approximately 6.6 to 7.2 mg DO/L during the death phase. This allows the assumption to be made that the choice of the death phase was incorrect and that the death phase had not commenced yet. The dark period DO values were considered to be the baseline, where the increase above the baseline was considered to be the net DO production by the algae (photosynthesis – respiration). However, this was only applicable for the linear growth phase.

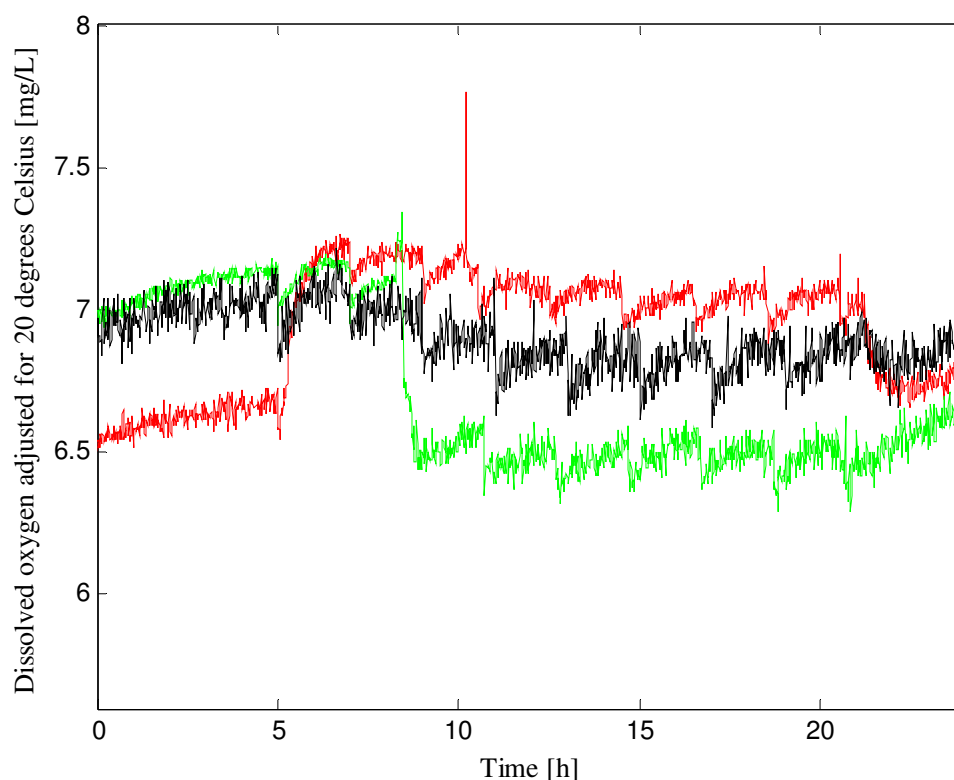


FIGURE 5.13 THE GRAPH SHOWS THE CHANGE IN DISSOLVED OXYGEN CONCENTRATION IN THE BIO-REACTOR FOR DIFFERENT GROWTH PHASES: LINEAR PHASE (—), STATIONARY PHASE (—) AND DEATH PHASE (—).

## 5.7 ALGAL DRY MASS AND LIPID CONTENT

The dry mass of the algae was determined to be 0.1234 g. It is important to note that during the freeze drying process, the algal mass was blown out of the petri dish, resulting in some loss of algal mass. However, most the algal mass was recovered.

Total lipids were found to be 4.46 % of the algal dry weight. Neutral lipids were not measured for the reasons stated in Section 4.7.

## 5.8 LIMITING FACTORS OF ALGAL GROWTH

As the macronutrients were not fully depleted, it is unlikely that N, P or C were the limiting factors of algae growth and ultimately the cause of algae death. For this batch experiment the pH was unfavorably low resulting in a reduced CO<sub>2</sub> feeding and hence possibly an insufficient supply of carbon. As the wastewater was obtained from the influent and clarifier of a wastewater treatment plant, it should provide micronutrients. At the same time this water also provided significant amount of bacteria as shown in Figure 5.14. These bacteria were likely to compete with the algae and could have contributed to micronutrient depletion. Furthermore, shear on the algae due to mixing may have contributed to the death of some of the cells. In terms of temperature, the culture experienced slightly lower temperatures (18-21°C on average) compared to their optimal culturing temperature (21-24°C), which may have reduced the growing activity. Figure 5.15 shows *Chlorella vulgaris* under a microscope at 1000x magnification. The image was taken after the batch experiment concluded. Here it becomes evident that there were not many healthy algal cells in the wastewater. In comparison, the small picture shows *Chlorella vulgaris* in a healthy state grown in MBL.



FIGURE 5.14 BACTERIA COLONIES AFTER 48 HOURS FROM A MUNICIPAL SAMPLE ONE DAY 10 AFTER INOCULATION ON DIFCO<sup>MT</sup> NUTRIENT AGAR.

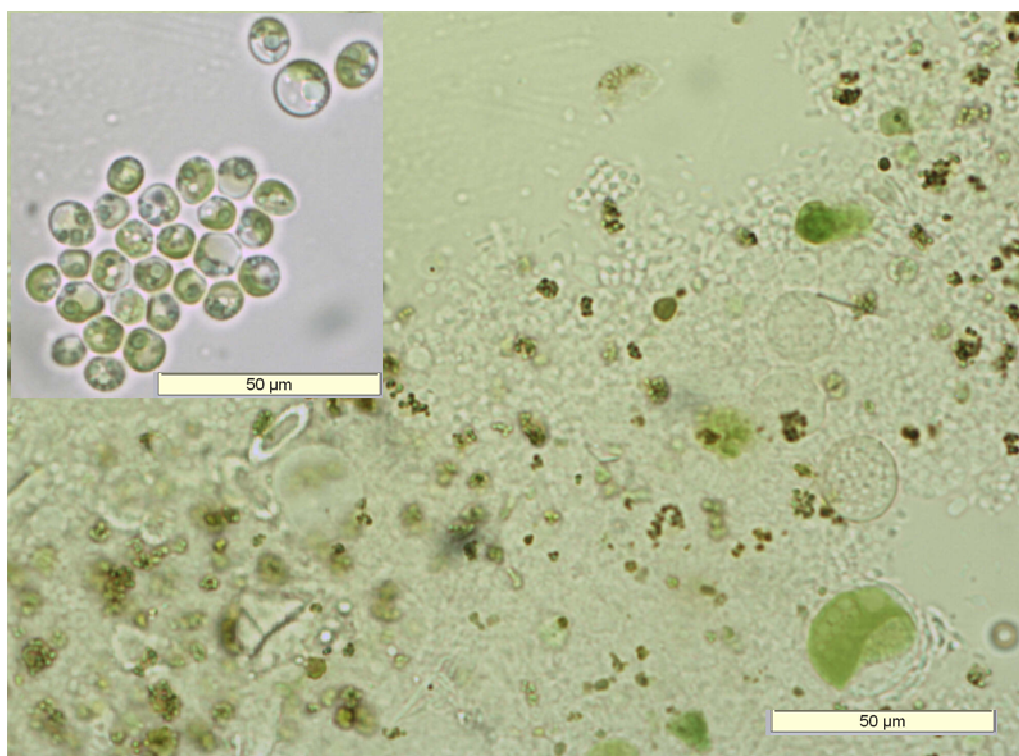


FIGURE 5.15 *CHLORELLA VULGARIS* IN MUNICIPAL WASTEWATER (AFTER THE BATCH EXPERIMENT CONCLUDED) UNDER 1000 x MAGNIFICATION. IN COMPARISON HEALTHY *CHLORELLA VULGARIS* CELLS IN MBL UNDER 1000 x MAGNIFICATION (SMALL PICTURE).

## 5.9 SUMMARY

This research has given insight into the algal growth rate in the municipal wastewater along with nutrient depletion rate of 1.59 mg TN/L/d and 0.51 mg PO<sub>4</sub>-P/L/d. Phosphorous was found to be much faster depleted than initially expected. The online measurements including pH and dissolved oxygen largely failed to indicate different algal growth patterns in this wastewater. The lipid content for this wastewater was found to be 4.48 %. There was evidence for significant bacterial growth which may have contributed to the poor performance of algal growth. In addition, the pH was found to increase only slowly resulting in a shorter CO<sub>2</sub> feeding period. The reduced feeding period may have lead to insufficient carbon supply to achieve higher algal growth.

## **CHAPTER 6      NUTRIENT REMOVAL AND LIPID PRODUCTION OF *CHLORELLA VULGARIS* MICROALGAE GROWN IN DAIRY WASTEWATER**

This chapter discusses the results of nutrient removal, growth behavior, fluctuations of pH and dissolved oxygen and lipid production of *Chlorella vulgaris* microalgae in wastewater obtained from a lagoon at a dairy farm. The water used for this experiment was pumped from the bottom of the lagoon. There were two batch experiments performed on this wastewater. The first experiment provided some indication of the general performance of the algae in the dairy wastewater, whereas the second batch experiment served for in-depth data analysis. There was no nutrient data collected for the first batch experiment due to a breakdown of the Ion Chromatography system.

### **6.1    BATCH EXPERIMENT 1 (PRELIMINARY EXPERIMENT)**

Wastewater for the first batch experiment was collected from a wastewater lagoon of a dairy farm. Due to its high turbidity (see Figure 6.1), the wastewater was filtered twice and diluted with distilled water. The dairy water used had a 5-day BOD of 126 mg/L and a 5-day BOD of 36 mg/L after dilution with distilled water. The nutrients in the wastewater had a concentration of 105.5 mg TN/L, 4.2 mg NO<sub>2</sub>-N/L, 0.006 mg NO<sub>3</sub>-N/L and 13.9 mg PO<sub>4</sub>-P/L, which resulted in a TN:P ratio of 38:5. The ratio was then adjusted to 10:1 with additional nitrogen as stated in Section 3.2.1. Due to the failure of the Ion Chromatography system, no data of nutrient changes could be obtained.

2-hourly CO<sub>2</sub> was supplied for 5 seconds from day 4 after inoculation. The pH generally remained low which meant that longer CO<sub>2</sub>-feeding-periods would have resulted in a significant amount of base addition. The pH tolerance was also increased to  $\pm 0.5$ .



According to spectrometry data and suspended solids measurements, the algae appeared to have entered an exponential growth phase during the first day. The growth quickly slowed down and the algae experienced a linear growth rate between day 2 and 3, followed by a stationary growth phase until approximately day 7. Signs of the death phase started to develop 7 days after inoculation. The correlation coefficient of the optical density and suspended solids was poor with  $R^2 = 35.1$ . The wastewater and *Chlorella vulgaris* remained brownish-green to brown at all times. Turbidity could have affected the suspended solids and optical density measurements and resulted in poor measurements resulting in low correlation between the two.



FIGURE 6.1 2.5 x DILUTED DAIRY WASTEWATER IN BIO-REACTOR

## 6.2 BATCH EXPERIMENT 2 - RAW WASTEWATER CHARACTERISTICS

The wastewater obtained from the dairy farm lagoon had the characteristics shown in Table 6.1. Total nitrogen of 302.4 mg/L, phosphorous of 38.1 mg/L, TOC of 44.9 mg/L and a 5-day BOD of 126 mg/L. Due to time constraints the 5-day BOD reading was adopted from the first dairy batch experiment. As this water was collected after a rain event (runoff water was able

to enter the lagoon), it is likely that the water was more diluted and would have therefore resulted in a slightly lower 5-day BOD.

The same wastewater/distilled water mix as for the first dairy batch experiment was adopted (see Table 6.2) with 1 L dairy water and 2.5 L distilled water. This was primarily due to the high turbidity of the wastewater. Therefore the diluted water was not chosen based on nutrient ratios but was rather based on reducing turbidity. Table 6.2 also shows that the N:P ratio was 79:10, which was close on an optimum of 8-16:1 and was therefore not further adjusted. The final composition of the dairy wastewater-distilled water-growth medium was 86.4 mg TN/L, 10.9 mg P/L, 12.8 mg TOC/L and a 5-day BOD of 36 mg/L. Even though the wastewater was filtered with 0.45  $\mu\text{m}$  filter paper and the food source for the bacteria was held low, there was still a significant risk of large amounts of bacteria present in the water. The reason for that is the set-up of the lagoon. The water was taken from the bottom of the lagoon through a pumping system. The water from the lagoon was likely to have been there for a substantial amount of time, building up large colonies of bacteria. It is impossible to filter out all bacteria. Section 6.8 further discusses this matter.

According to the dairy farm manager, there is likely to be some antibiotics and cleaning products present in the wastewater. Antibiotics are used in the dairy industry to control gastrointestinal microbes in livestock.

TABLE 6.1 WASTEWATER CHARACTERISTICS OBTAINED FROM THE DAIRY WASTEWATER

Mixes		
	Dairy	Distilled Water
TN (mg/L)	302.35	0
P (mg/L)	38.06	0
TOC (mg/L)	44.93	0
BOD (mg/L)	126	0

TABLE 6.2      SELECTED WASTEWATER MIX WITH NUTRIENT VALUES AND ORGANIC STRENGTHS

	Dairy	Distilled Water		
	Parts			
	2	5		
	Amounts		Total	TN:P Ratio
TN (mg/L)	86.386	0	86.386	7.9
P (mg/L)	10.874	0	10.874	1
TOC (mg/L)	12.837	0	12.837	-
BOD (mg/L)	36.000	0	36.000	-

### 4.3 GROWTH CHARACTERISTICS

This batch experiment did not show any obvious growth phases (Figure 6.2). The algae appeared to have experienced a lag phase during the first day and then slowly increased in optical density and suspended solids mass. Considering the suspended solid measurements, a stationary growth phase may have been entered around day 11. However, this was not evident with optical density. The death phase was assumed to have been entered after day 14. Again, there were no obvious signs that the death phase had started. A reason for the less prominent differentiations between the growth phases may have been relatively low algal masses and optical densities observed.

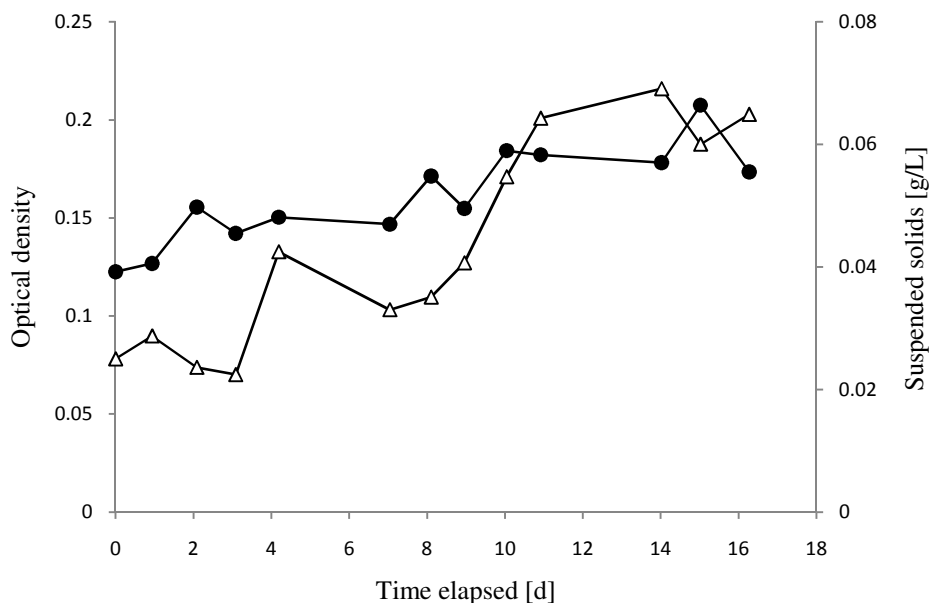


FIGURE 6.2 ALGAL GROWTH IN DAIRY WASTEWATER WAS MONITORED BY OBSERVING DAILY CHANGES BY MEASURING THE ABSORBANCE AT THE OPTICAL DENSITY (●) AT 505 NM WITH A SPECTROPHOTOMETER (JENWAY 6705 UV/VIS.) AND SUSPENDED SOLIDS (Δ)

Both, the optical density and the suspended solids readings appeared to have given very similar results. A relationship between suspended solids and optical density can be seen in Figure 6.3. The relationship between the two can be expressed as shown in Equation 6.1.

$$\text{suspended solids} = 0.561 \text{ optical density} - 0.047 \quad (R^2 = 0.63) \quad (\text{Eqn. 6.1})$$

It is possible the water's brown colour may have affected the spectrophotometer readings. In addition, a significant amount of very small particles in the water may have contributed to a false reading for the suspended solids measurement. A blank correction to account for the existing particles in the wastewater was not performed as an additional reactor undergoing the same amount of evaporation losses would have been required. This could also explain why it was difficult to identify individual growth phases in this water.

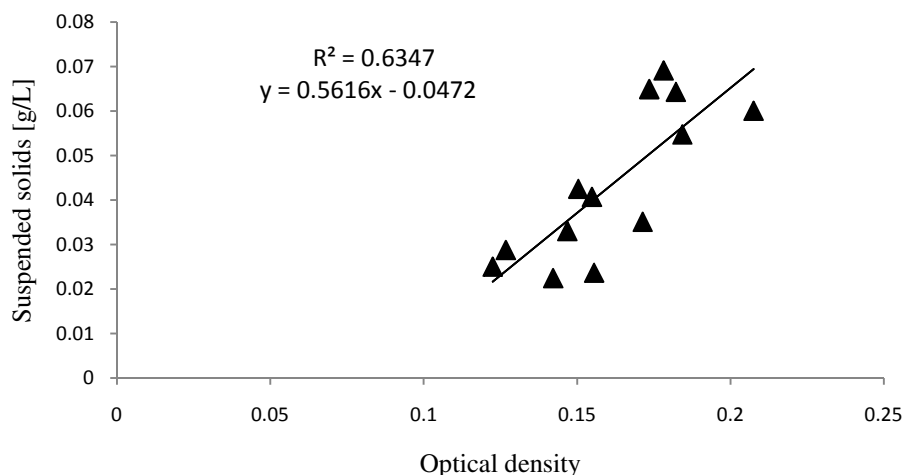


FIGURE 6.3 RELATIONSHIP BETWEEN SUSPENDED SOLIDS MEASUREMENTS AND OPTICAL DENSITY AT 505NM USING DAIRY WASTEWATER.

Figure 6.4 shows filter paper from the suspended solids measurements. It shows that throughout the experiment there was a strong discoloration of the water; nevertheless an increase in mass can be detected. A sample viewed under a microscope confirmed that the mass was indeed mainly *Chlorella vulgaris* microalgae, albeit many of the cells were destroyed and torn apart as the algae entered the death phase. The alive algae cells showed their typical green colour, where dead cells lost most of their green colour and appeared brown. Therefore it can be concluded that the brown discoloration on the filter papers was due to dead algae cells and the colour of the wastewater.

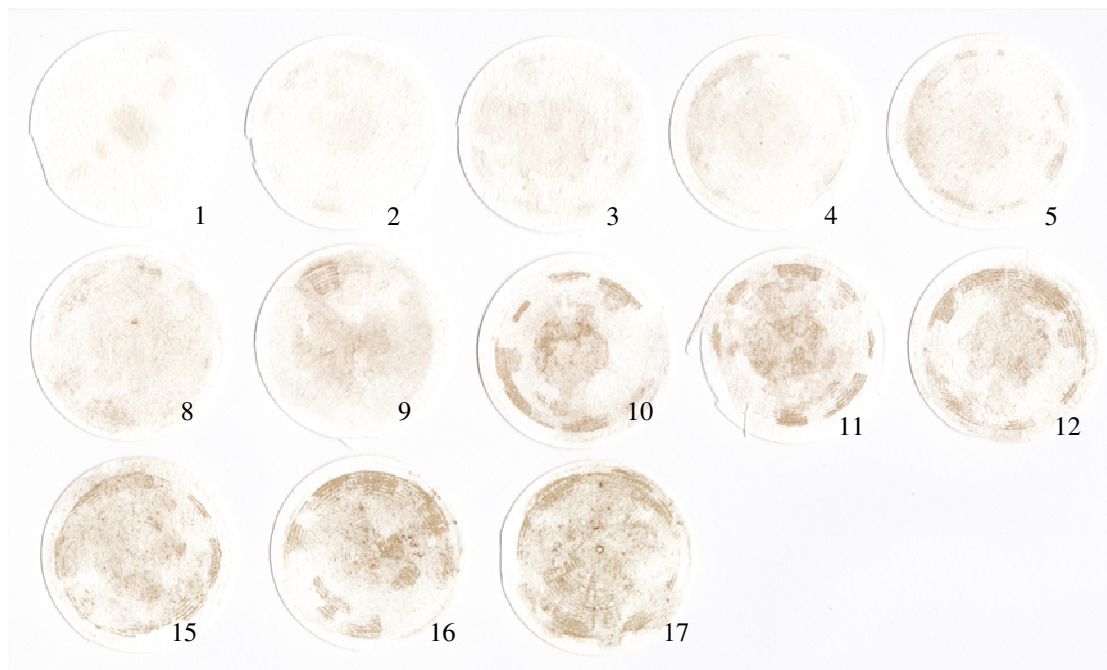


FIGURE 6.4 FILTER PAPER COLLECTED OVER THE GROWTH PERIOD IN DAIRY WASTEWATER, WHERE THE NUMBERS REPRESENT THE DAY OF FILTRATION.

## 6.4 NUTRIENT REMOVAL

Figure 6.5 shows the change in nitrogen concentration in the wastewater during the culturing period. Total nitrogen decreased by 1.73 mg/L/d. The algal growth in terms of suspended solid increase was 2.88 mg SS/L/d. Assuming all the suspended solids were from algal cells, the specific growth rate of algae can be deduced as 0.6 TN/SS. Organic-N and  $\text{NH}_4\text{-N}$  decreased at a rate of 4.72 mg/L/d with a specific growth rate of 1.64 (Org-N +  $\text{NH}_4\text{-N}$ )/SS. Ammonia concentrations did not significantly reduce until day 4 after inoculation. The quick ammonia reduction also coincides with the nitrite increase as shown in Figure 6.6. It is possible that *Nitrosomonas* nitrifying bacteria were present (TWPC n.d.) and began converting ammonia to nitrite from day 4. Nitrite continued to fluctuate between 1 and 4 mg/L. Figure 6.6 also shows how nitrate started to increase from day 8. Therefore it was assumed that *Nitrobacter* bacteria (TWOC n.d.) were present that converted the nitrite to nitrate just a few days later. Nitrate nitrogen increased at a rate of 4.48 mg/L/d at a specific growth rate of 1.56  $\text{NO}_3\text{-N}$ /SS.

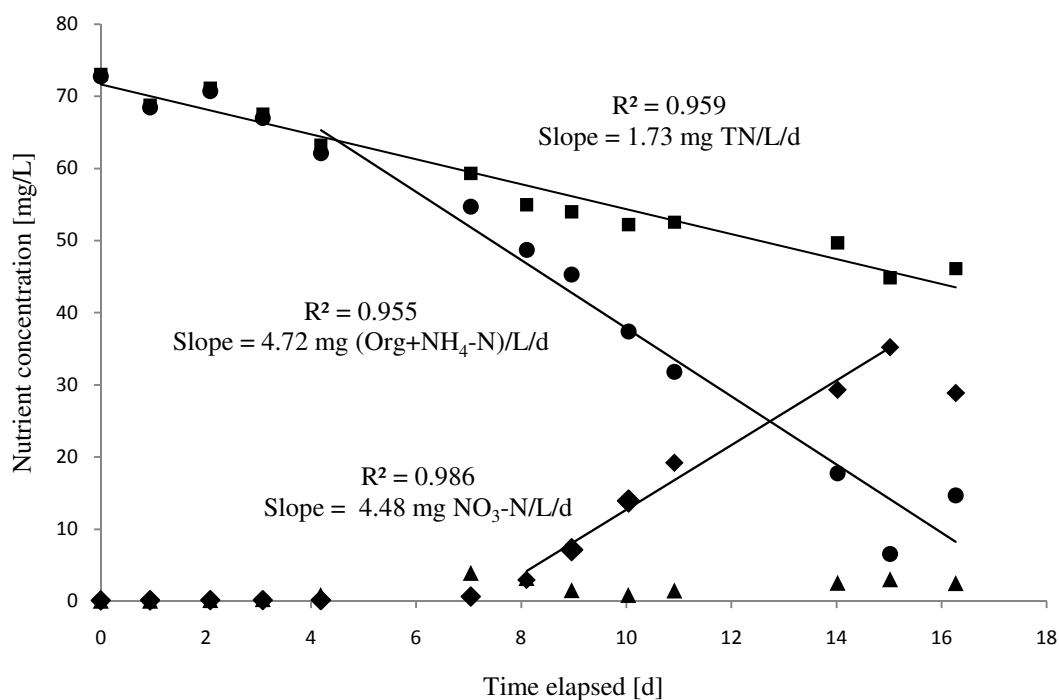


FIGURE 6.5 THE GRAPH SHOWS THE CHANGE IN NITROGEN CONCENTRATION IN FORM OF ORG-N + NH<sub>4</sub>-N (●), TN (■), NO<sub>2</sub>-N (▲), NO<sub>3</sub>-N (◆) IN DAIRY WASTEWATER.

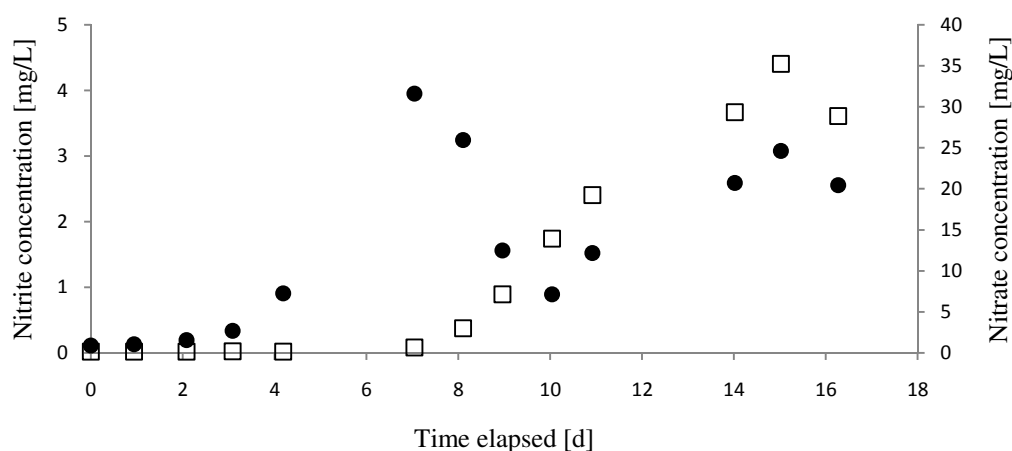


FIGURE 6.6 CHANGE IN NITRITE (●) AND NITRATE (□) OVER THE GROWTH PERIOD.

Phosphate-P was not found to be depleted. But since algal growth was evident and therefore P-reduction should have been present, it is possible that the collected measurements had some error. Figure 6.7 even shows a slight increase in phosphate with 0.02 mg/L/d, which was believed to be impossible.

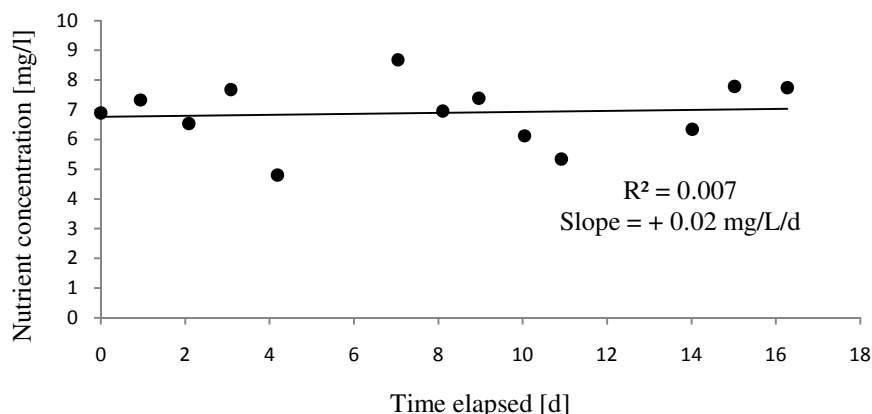


FIGURE 6.7  $\text{PO}_4\text{-P}$  CHANGE WITH TIME USING DAIRY WASTEWATER.

## 6.5 pH CHANGE WITH TIME

In addition to the continuous 10 ml/min  $\text{CO}_2$  supply,  $\text{CO}_2$  was provided every two hours for 16 hours from 5 am to 9 pm at a volume of 56 ml/min flow rate for 5 seconds from day 3. The pH tolerance was set to  $\pm 0.6$ , which was a slight increase of the  $\pm 0.5$  tolerance in the first batch experiment. Figure 6.8 shows the pH behaviour over the entire culturing period. There was a significant reduction in pH noticeable from day 8. This is also the time when nitrifying bacteria began to convert nitrite to nitrate. Nitrification causes a reduction in pH causing more acidic pH levels (TWOC n.d.). This could explain why pH reduced to some extent. However, the pH was able to be kept above threshold through frequent base addition from day 8. On day 12 the base piping ruptured and the base pumping was consequently stalled. The increase in pH from that day was due to manual sodium hydroxide addition to avoid very low pH levels.



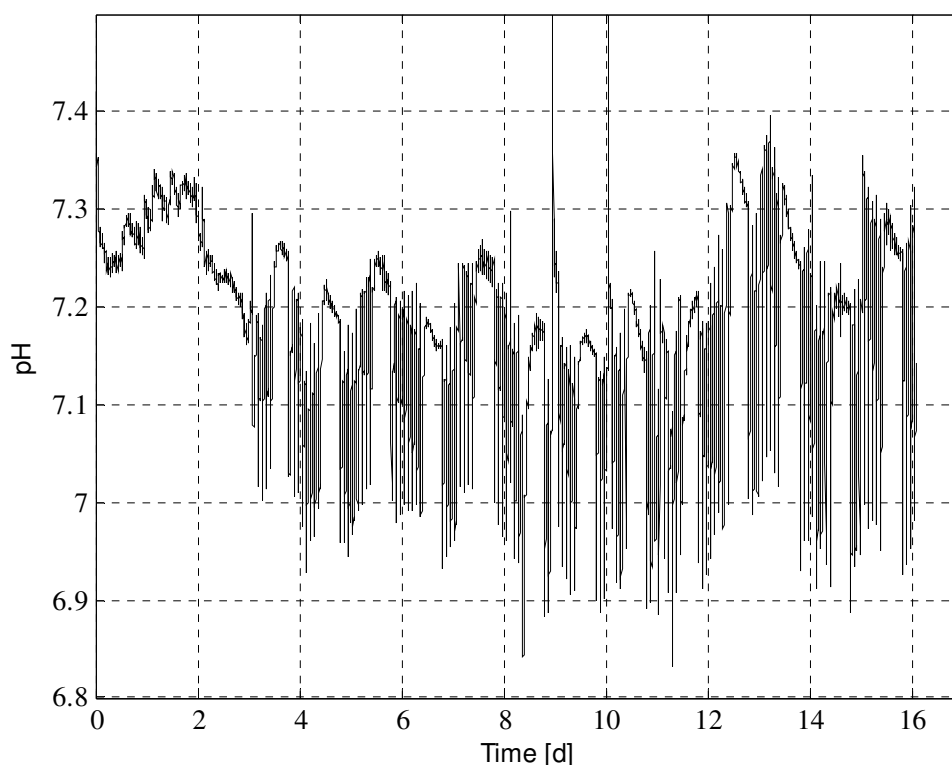


FIGURE 6.8 pH WAS MONITORED EVERY MINUTE FOR 16 DAYS IN DAIRY WASTEWATER

Figure 6.9 shows the pH changes that occurred during the linear and stationary growth phases for 24 hours. The lag and death phase was not pronounced enough and could therefore not be used in a reliable way and hence was omitted.

Both the stationary and linear phase demonstrates the algae's growth between  $\text{CO}_2$  supplies. During the linear growth phase, the slope was 0.114 pH unit/h where during the stationary phase the slope was 0.149 pH unit/h. This indicates that a larger biomass was available for the consumption of  $\text{CO}_2$  during the stationary phase. For both phases, the slope was slightly shallower for the first two hours until when the first  $\text{CO}_2$  addition occurs, possibly due to the adjustment of the algae to the light period.

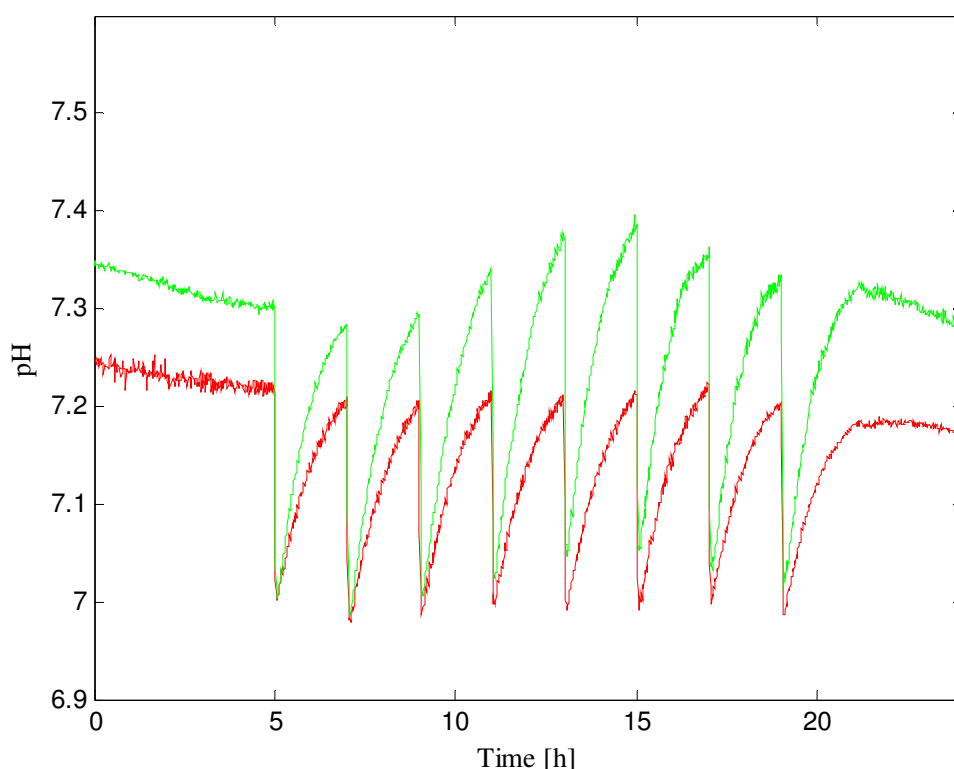


FIGURE 6.9 THE GRAPH SHOWS THE pH INCREASE DUE TO  $\text{CO}_2$  CONSUMPTION BY THE ALGAE DURING THE LIGHT PERIOD AND THE DECREASE OF pH DUE TO  $\text{CO}_2$  ADDITION FOR DIFFERENT GROWTH PHASES: LINEAR PHASE (—), STATIONARY PHASE (—).

## 6.6 DISSOLVED OXYGEN CHANGE WITH TIME

Figure 6.10 shows the concentration of dissolved oxygen for the 16 day culturing period. The bottom graph gives an indication for light and dark periods. The oxygen production remained largely unchanged until about day 6. From there oxygen production reduced significantly until about day 11. Due to time constraint during that period, the DO meter was not re-calibrated. This could have caused the artificial reduction in dissolved oxygen. Calibration occurred on day 11 and dissolved oxygen was shown correctly. However, the DO readings showed a slight reduction from the readings obtained on the first six days after inoculation. This leads to the assumption that the death phase of the algae commenced and less oxygen was produced during photosynthesis. Alternatively, the reduced DO readings may have been associated with the increased bacterial mass. Nitrifying bacteria are aerobic

and hence utilise oxygen for nitrification. Their activity could have increased the total oxygen demand in the bio-reactor.

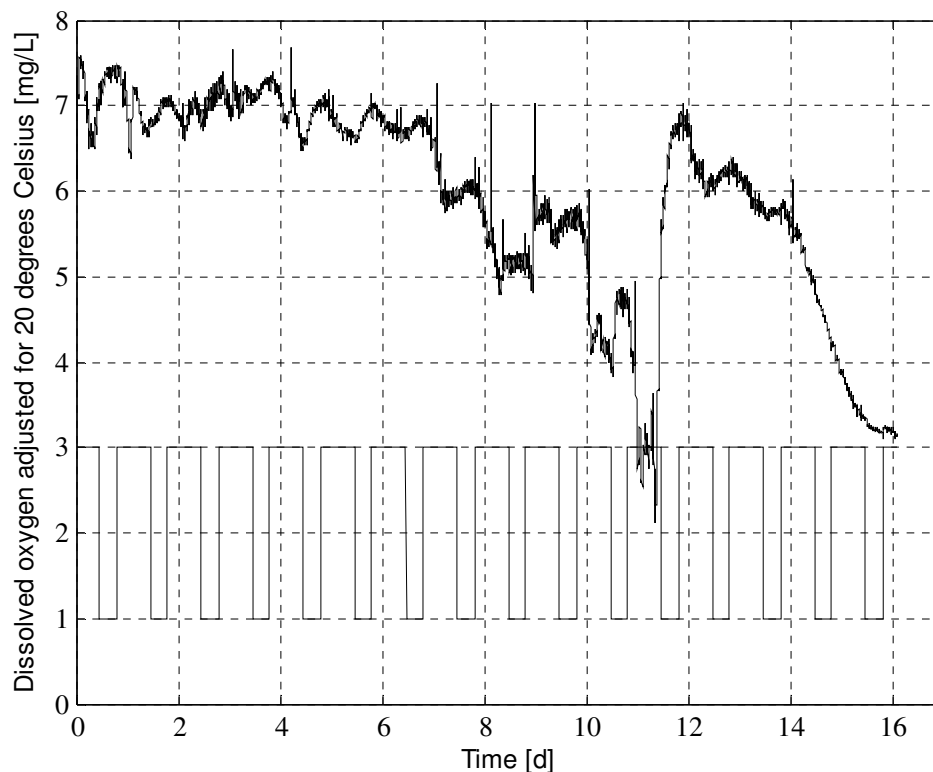


FIGURE 6.10 DISSOLVED OXYGEN WAS ADJUSTED TO 20 DEGREES CELSIUS AND MONITORED EVERY MINUTE FOR 16 DAYS. THE BOTTOM GRAPH INDICATES LIGHT (PEAKS) AND DARK (VALLEYS) PERIODS.

Figure 6.11 shows the change in dissolved oxygen for the linear and stationary growth phase over 24 hours. The oxygen levels in the linear growth phase were much higher than the oxygen levels in the stationary phase for reasons stated above. Interestingly, the oxygen levels generally reduced during the light period. A reason for this behaviour could not be identified.

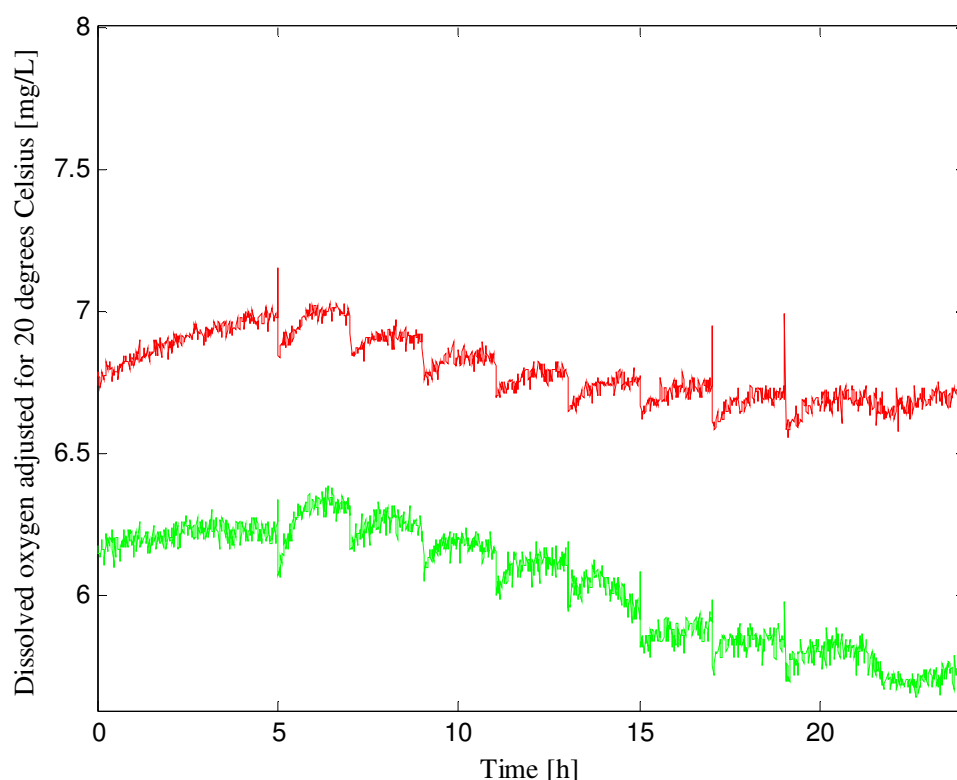


FIGURE 6.11 THE GRAPH SHOWS THE CHANGE IN DISSOLVED OXYGEN CONCENTRATION IN THE BIO-REACTOR FOR DIFFERENT GROWTH PHASES: LINEAR PHASE (—), STATIONARY PHASE (—).

## 6.7 ALGAL DRY MASS AND LIPID CONTENT

The dry mass of the algae was measured to be 0.079 g. Unfortunately, due to technical error, up to 50 % of the algal dry mass was lost. In addition to the dairy wastewater sample was mixed with the municipal wastewater algal dry mass. Fortunately the municipal sample was lighter in colour and was made out of dried algal chunk. This allowed an almost complete recovery of the municipal sample. However, small municipal particles were mixed with the dairy sample. Therefore, up to 50 % of the dairy sample was made up with municipal algal cells. The total lipid content was measured to be 4.071 % of the algal dry mass. Neutral lipids were not measured.

## 6.8 LIMITING FACTORS OF ALGAL GROWTH

It is possible that a significantly reduced amount of ammonia could have played a significant part of the algal death. The reduction of ammonia was likely to be due to algal uptake and through nitrification activity by nitrifying bacteria. From previous experiments in the dissertation and from the literature it was increasingly evident that ammonia is the algae's preferred form of nitrogen. Figure 6.12 and 6.13 show a petri dish with CM0007 MacCONKEY Agar. A small sample of the dairy wastewater, 5 days after inoculation was placed on the agar and then stored at 37°C in an incubator for 24 hours. Figure 6.12 and 6.13 show a brown discoloration of the originally red agar. Several bacterial species were present in this water sample, and were most likely non-lactose fermenting members of the *Enterobacteriaceae* family – *E. coli* was not identified. Several colonies consistent with the appearance of *Staphylococci* are also present as shown in Figure 6.13 (pers. comm. M. Boddington, October, 2010).

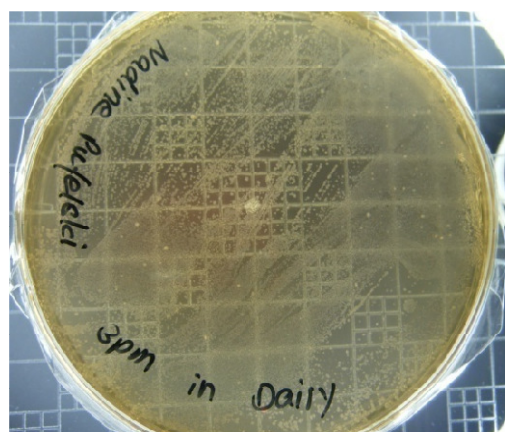


FIGURE 6.12 *ENTEROBACTERIACEAE* BACTERIA ON CM0007 MacCONKEY AGAR AFTER 24 HOURS FROM DAIRY WASTEWATER ON DAY 5 AFTER INOCULATION

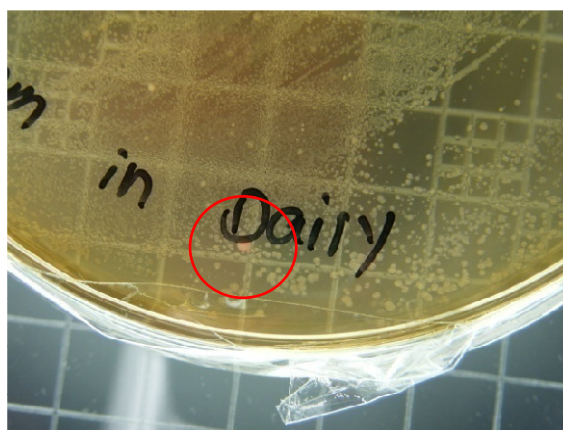


FIGURE 6.13 *STAPHYLOCOCCUS* SPECIES ON CM0007 MacCONKEY AGAR

After emptying the reactor, it was found that some of the algae attached to the walls of the reactor. This confirmed that algal growth was present, even though it was not readily observable during the batch experiment. The algae's preference to attach to the reactor suggests that the light penetration was low in the reactor. The light conditions at the reactor walls would have provided the most suitable light environment for the algae. It can be deduced that the amount of light was too low for the algae in order to grow readily and hence it could have contributed to the poor algal mass collected.



FIGURE 6.14 ALGAL GROWTH ON THE REACTOR WALLS, OBSERVED AFTER EMPTYING OF THE REACTOR.

## 6.9 SUMMARY

This research has given insight into the algal growth rate in the dairy wastewater along with nutrient depletion rate of 1.73 mg TN/L/d and + 0.02 mg PO<sub>4</sub>-P/L/d, with the specific growth rate of 0.6 TN/SS. There was some evidence that nitrification occurred in the reactor, converting ammonia to nitrite and nitrate. As algae appear to prefer ammonia as a nitrogen source, bacterial activity would have competed with the algae for ammonia. In addition it was found that the light penetration into the reactor was very low. This would also have significantly contributed to poor algal growth. The total lipids equated to 4.07 % of the algal dry mass.

## CHAPTER 7      COMPARATIVE STUDY BETWEEN DIFFERENT WASTEWATERS

This chapter compares the performance of *Chlorella vulgaris* in terms of algal growth, nutrient depletion, CO<sub>2</sub> uptake abilities and lipid production in the three analysed wastewaters. Lipid results of this research are also compared with results obtained from external researchers.

### 7.1 COMPARISON OF GROWTH

Figure 7.1 shows three graphs of algal growth in nursery, municipal and dairy wastewater. It can be seen that nursery wastewater provided by far the largest growth. Municipal and dairy wastewater show similar growth curves. Possible reasons for the poorer performance in the latter two wastewaters may have been:

1. More turbidity in municipal (due to the presence of yeast effluent) and dairy wastewater; and
2. A large bacterial load that competed with the algae for P and further increased turbidity through bacterial growth.

Interestingly, the suspended solids measurements in the nursery and municipal graphs were above the optical density curves, where the order of curves was reversed for the dairy wastewater. This behaviour could indicate some measuring error in either suspended solids or optical density in the dairy wastewater or perhaps it could be a normal outcome when using different wastewaters. From observations it was found that algal growth was the lowest in the dairy wastewater.

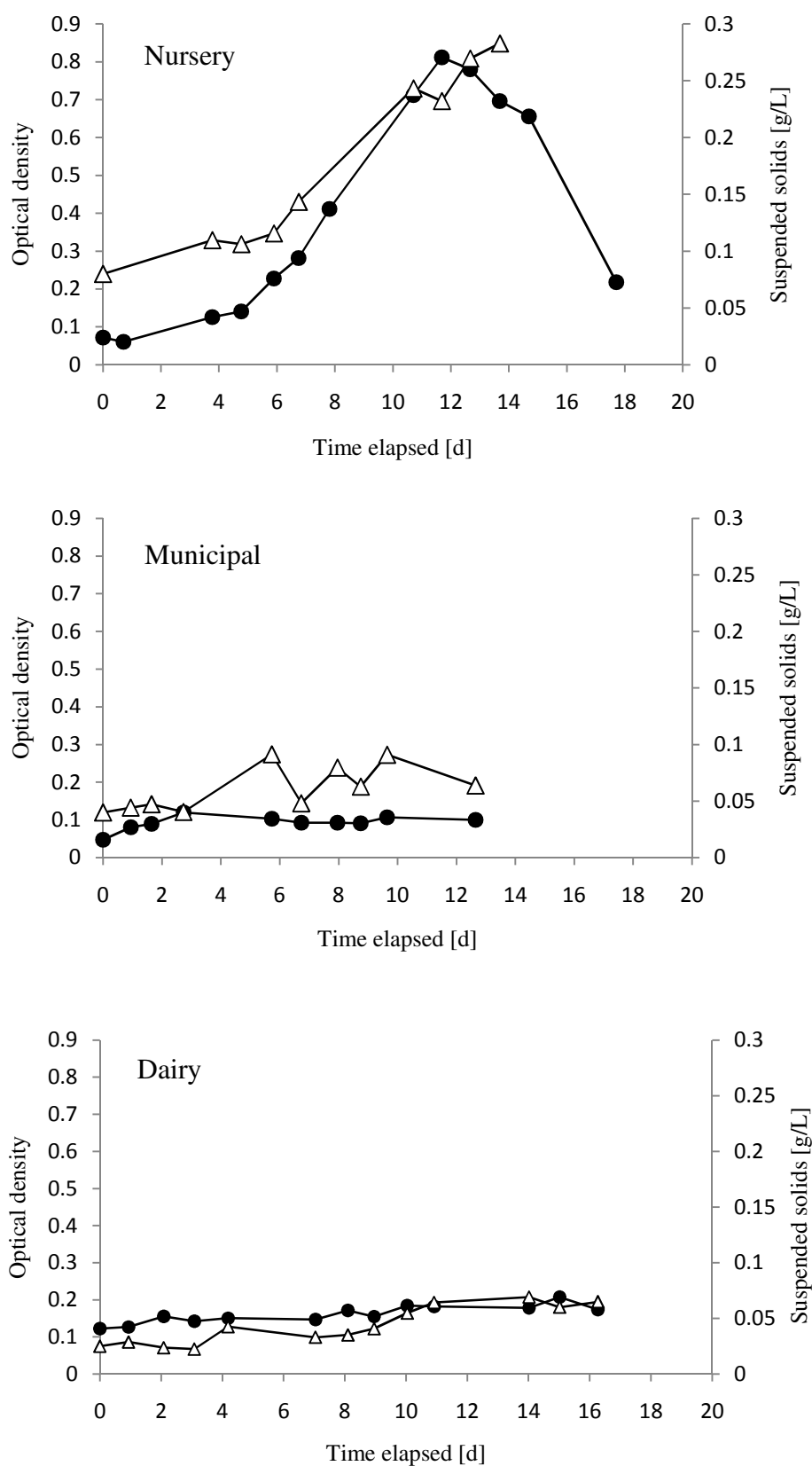


FIGURE 7.1 COMPARISON OF GROWTH BEHAVIOUR IN NURSERY, MUNICIPAL AND DAIRY WASTEWATER IN FORM OF OPTICAL DENSITY (●) AND SUSPENDED SOLIDS (Δ).



## 7.2 COMPARISON OF NUTRIENT UTILISATION

### 7.2.1 UTILISATION OF NITROGEN

The study has found that *Chlorella vulgaris* was able to grow in all three wastewaters and consequently reduced nitrogen. The largest nitrogen reduction rates were found in the nursery wastewater with a total nitrogen removal rate of 2.64 mg/L/d, followed by dairy and municipal wastewater with removal rates of 1.73 mg/L/d and 1.59 mg/L/d respectively. Therefore nursery wastewater allowed the most effective nitrogen removal as shown in Figure 7.2. Organic nitrogen and ammonia nitrogen removal is shown in Figure 7.3. It can be seen that dairy wastewater showed the most significant reduction of Org. +  $\text{NH}_4$  -N with a rate of 4.72 mg/L/d. But at the same time, dairy wastewater did not cause the largest total nitrogen reduction. The reason for that lies in the nitrifying bacteria that were present in large numbers in the dairy wastewater. They utilised ammonia to change it into nitrite (not shown here) and finally nitrate. This can be confirmed in Figure 7.4 where dairy wastewater showed increased nitrate rates. This allows the conclusion to be made that dairy wastewater had significantly larger amounts of nitrifying bacteria than nursery or municipal wastewater.

As nitrification was evident in all three wastewaters, it was difficult to determine how much ammonia-N and nitrate-N was really removed by the algae and which proportion was removed by bacteria.

It is important to note that some of the removal rates in Figure 7.2 to 7.5 lasted for the entire culturing period, where others only lasted for a few days. For that reason it was possible to reduce organic nitrogen and ammonia nitrogen at a rate of 4.72 mg/L/d and increase nitrate nitrogen at a rate of 4.48 mg/L/d and still achieve an overall nitrogen removal rate of 1.73 mg/L/d.

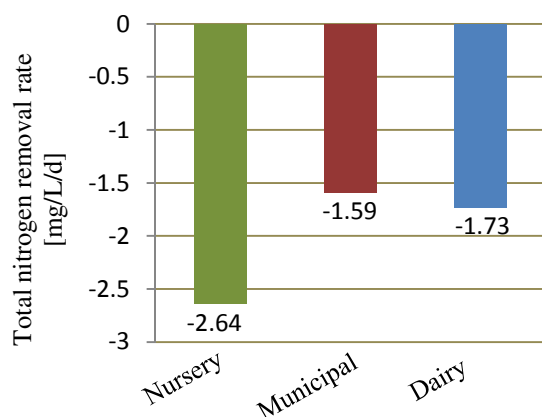


FIGURE 7.2 COMPARISON OF TOTAL NITROGEN REMOVAL RATES

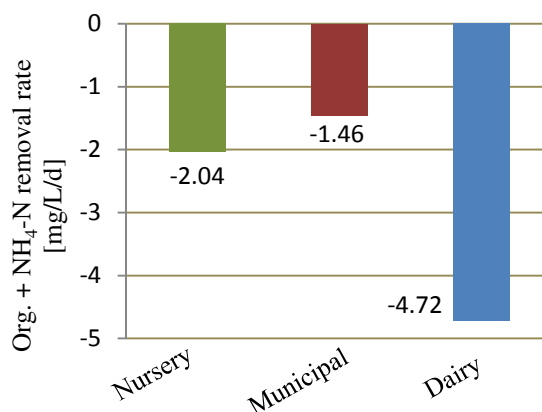


FIGURE 7.3 COMPARISON OF ORG. + NH<sub>4</sub> NITROGEN REMOVAL RATES

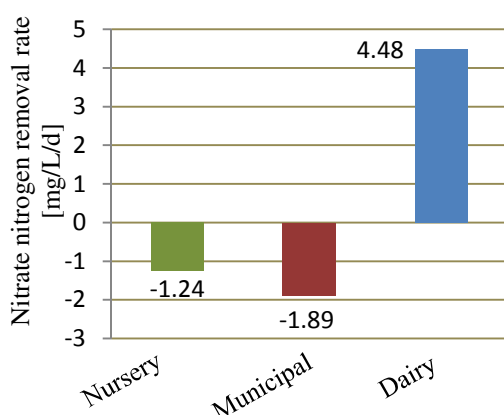


FIGURE 7.4 COMPARISON OF NITRATE NITROGEN REMOVAL RATES

## 7.2.2 UTILISATION OF PHOSPHOROUS

Figure 7.5 shows the phosphorous removal rate in all three wastewaters. Whilst nursery wastewater showed a typical removal rate based on the nitrogen removal (at a N:P ratio between 8:1 and 16:1), the municipal wastewater showed an unexpectedly high phosphorous removal rate (N:P ratio of 3:1). This is a very promising reaction since it is the increased phosphorous level in water bodies that can cause elevated risk of experiencing eutrophication. However, this behaviour requires further repetitive experimental work before it can be confirmed as a common performance. The first municipal batch experiment showed an increase in phosphorous which is believed to be a measuring error. Unfortunately dairy water also showed no reduction in phosphorous, but instead a small increase. As an increase

in phosphorous levels is impossible, it is likely that a measuring error has caused an artificial phosphorous increase. The reason for the lack of phosphorous usage is thought to be due to the very small growth rate of *Chlorella vulgaris* in the turbid dairy wastewater.

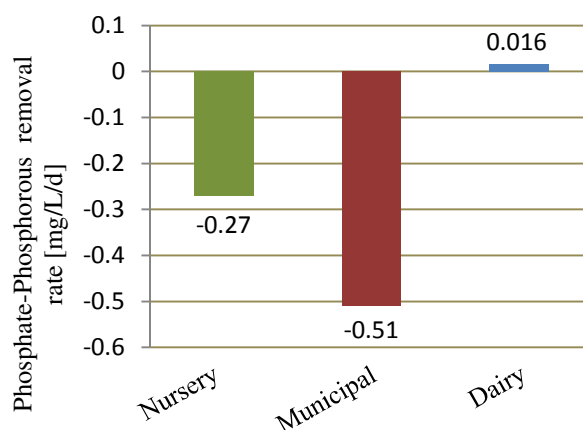


FIGURE 7.5 COMPARISON OF PHOSPHATE-PHOSPHOROUS REMOVAL RATES

### 7.3 COMPARISON OF CO<sub>2</sub> UPTAKE ABILITIES

Abilities of CO<sub>2</sub> uptake were based on pH fluctuations due to CO<sub>2</sub> feeding and acid/base addition as well as the amount of pH increase between CO<sub>2</sub> feedings (a large increase was desired). Based on the pH behaviour, CO<sub>2</sub> feeding durations and pH tolerances had to be adjusted to avoid large additions of acid or base. Only nursery wastewater was able to remain between the chosen pH tolerance of  $\pm 0.3$ , where the tolerance had to be increased to  $\pm 0.6$  for municipal and dairy wastewater. In addition, the feeding duration had to be reduced to 5 seconds for the municipal and dairy wastewater to avoid pH levels falling below the tolerance level. The reason for the low pH in both wastewaters may have been the presence of nitrification which decreases pH levels. An attempt was made to increase the pH permanently for these two wastewaters in supplying a larger than necessary amount of sodium hydroxide (the amount was not measured; only individual NaOH pellets were added). Unfortunately the pH reduced again within just a few hours. This confirms the pH reduction due to nitrifying bacteria activity.

Therefore in this study it was found that CO<sub>2</sub> uptake ability was largest in the nursery wastewater, most likely due to the smaller amount of nitrifying bacteria present. This means that nursery wastewater would be most suited for a carbon dioxide mitigation facility.

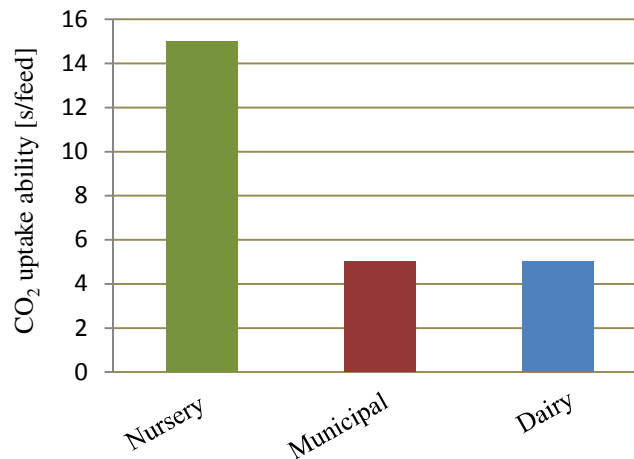


FIGURE 7.6 COMPARISON OF CO<sub>2</sub> UPTAKE ABILITIES

#### 7.4 COMPARISON OF LIPID PRODUCTION

Figure 7.7 shows total and neutral lipid measurements taken from this research and other external research. The external research results were taken from Table 2.2 in Chapter 2. All researchers shown in Figure 7.7 utilised *Chlorella vulgaris* microalgae. Total lipid masses obtained from this research are shown in Figure 7.8. It is important to note that neutral lipids were only measured for nursery wastewater and the first batch of municipal wastewater due to doubts regarding the accuracy of neutral and polar lipid separation techniques.

Municipal wastewater showed a total lipid content between 4.5 and 12.8 % of the dry weight. Not only did the first batch experiment perform better in terms of lipid production, there was also a significantly larger amount of lipids available by weight. The first batch experiment achieved 0.028 g where the second experiment achieved only 0.05 g.

The first batch of nursery wastewater achieved 25.5 % in total lipids with 7.6 % neutral lipids and the second batch achieved 9.7 % in total lipids and 4.1 % neutral lipids. As mentioned in

Chapter 4, it is likely that the lipid percentages for the second batch experiment were vastly underestimated due to large amount of algae harvested. For example, the first experiment achieved 0.026 g of total lipid weight, where the second resulted in 0.064 g in lipids.

Due to time constraints, lipid extraction for dairy wastewater was only performed on the first batch. Total lipids were found to be only 4.1 % with 0.003 g in total lipid weight. However, due to a technical error (see Section 6.7), the measured total lipid weight may have been significantly understated.

Assuming that the neutral lipid results for the nursery and municipal wastewaters were representative and assuming that all neutral lipids can be converted into FAME, the following calculation provides an approximate amount of wastewater required to produce 1 L of bio-diesel: The second batch of nursery wastewater achieved 0.0273 g neutral lipids where the first batch experiment of municipal wastewater achieved 0.0114 g. With a diesel density of 0.84 kg/L (pers. comm. T. Yusaf, October 2010), 840 g of neutral lipids are required for 1 L of fuel. That means about 108,000 L of nursery wastewater or 258,000 L of municipal wastewater are required to produce 1 L of bio-diesel. These numbers may appear non-feasible, but it is important to note that lipid extraction was done by hand and with very small amounts of algal mass. Therefore the potential experimental error could have been very large. In addition it must be noted that this experiment did not undergo any optimisation activities. Optimisation could result in significantly improved lipid amounts.

Generally it can be said that nursery wastewater performed best in terms of lipid production, followed by municipal dairy wastewater. This is the opposite of originally expected. Due to the expected large range of nutrients in the municipal wastewater and possibly elevated iron content, it was believed that the municipal water would result in vigorous algal growth. Toowoomba has elevated amounts of iron in their bore water sources which consequently ends up at the wastewater reclamation facility. Chisti (2007) found that algae perform well in the presence of iron. Possible reasons for the good performance in nursery wastewater may have been the composition of the water in terms of nutrients and possibly a lower bacterial load. As algae are plants, they might have been able to profit of the balanced composition of fertilizer nutrients. Another factor could have been induced stress on the algal cells due to

the absence of certain micronutrients. Algal cells store more lipids under certain stresses. The municipal and dairy wastewater performed at a lower lipid rate. Possible reasons may have been significant competition with bacteria, lower light intensities and toxins in the wastewater. However, none of these possible reasons could be verified due to research limitations.

All lipid results of this research were competitive with other research conducted in the past. In Figure 7.7 below, only De-Bashan et al (2002) used some type of wastewater (secondary municipal wastewater effluent). Unfortunately they achieved only a very small amount of total lipids for reasons not stated. All other external researchers used some type of artificial medium. Harris (2010) for example used MBL as a basic growing medium. The reason why the wastewater experiments in this research performed better than in the research conducted by Harris (2010) may be:

1. This research used larger culturing volumes which results in smaller measuring error and loss of lipids during extraction;
2. This research may have stressed the algae to a higher extent resulting in a larger accumulation of lipids in the algal cells; and
3. This research provided a relatively natural environment, providing habitat for algae and bacteria. Such environments often promote better growth and hence lipid production (per. comm. P. Pittaway, October, 2010).

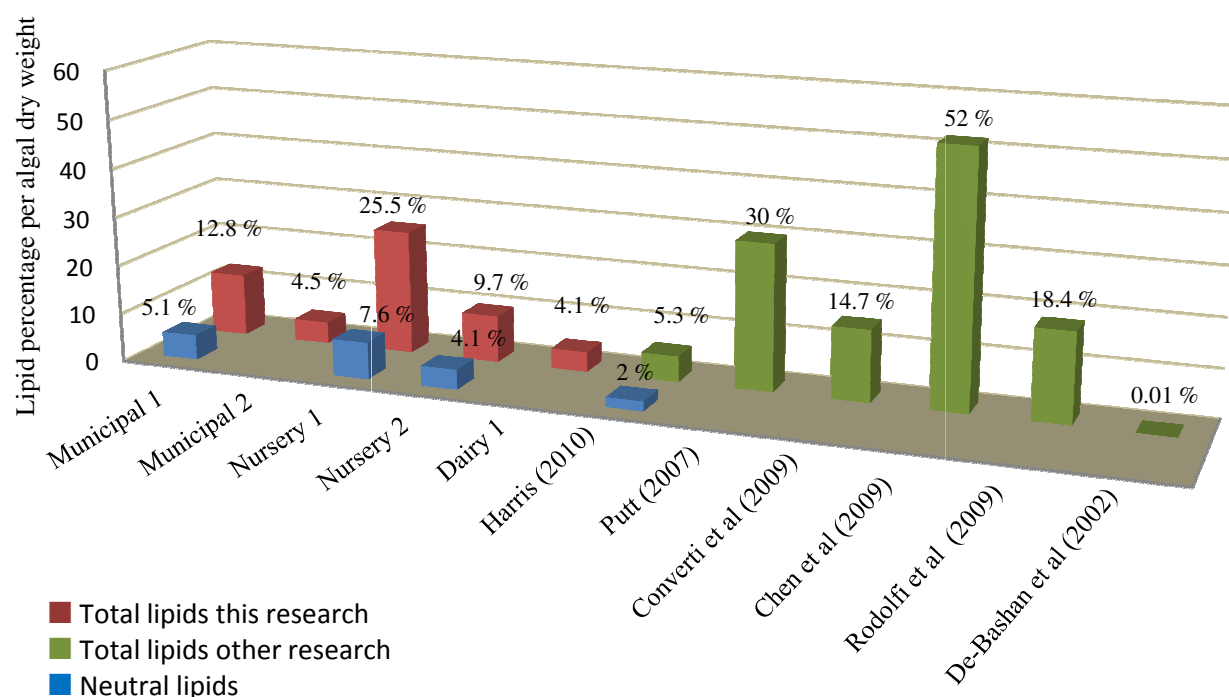


FIGURE 7.7 LIPID PERCENTAGES FROM THIS RESEARCH AND EXTERNAL RESEARCH

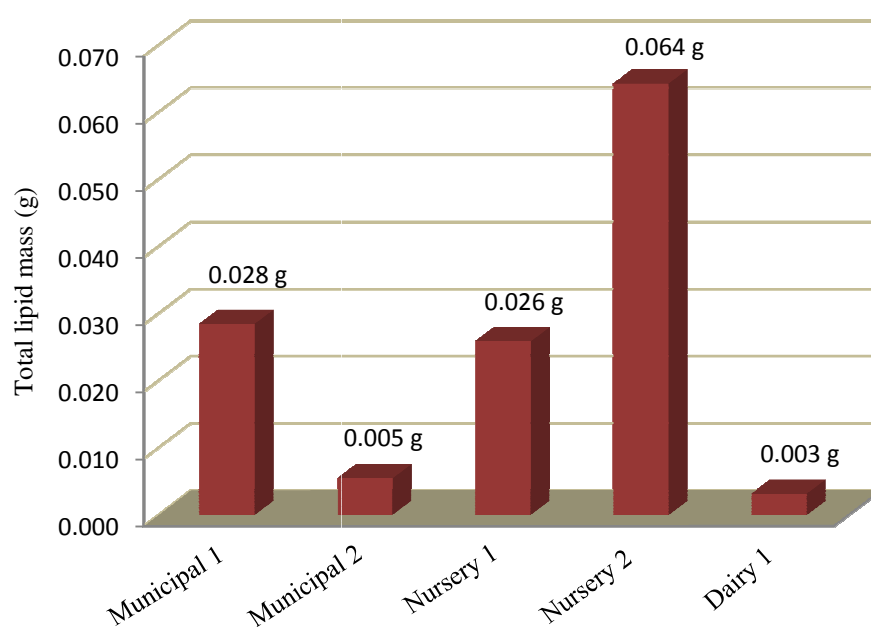


FIGURE 7.8 TOTAL LIPID MASSES OBTAINED FROM THIS RESEARCH

## 7.5 SUMMARY

This chapter compared the performances of *Chlorella vulgaris* in terms of nutrient removal, algal growth and lipid production in the three studies wastewaters. It was found that maximum growth was achieved in the nursery wastewater.

The nitrogen depletion rate was also the highest in nursery wastewater with 2.64 mg/L/d. The dairy wastewater appeared to have the largest bacterial load based on nitrate nitrogen increases. Phosphorous was mostly depleted in municipal wastewater at a rate of 0.51 mg/L/d which resulted in an unusually high phosphorous utilisation with a N:P ratio of 3:1.

Nursery wastewater showed the largest total lipid production with a maximum of 25.5 % total lipids of its dry weight. Municipal wastewater achieved 12.8 % total lipid production followed by dairy wastewater with 4.1 %. However, these results also contained experimental errors.



## CHAPTER 8 CONCLUSIONS AND FUTURE WORK

### 8.1 CONCLUSIONS

In this research, *Chlorella vulgaris* microalgae were used to uptake nutrients (in particular nitrogen and phosphorous), mitigate carbon dioxide and produce lipids for bio-diesel production using wastewater as a culturing medium. Because of the algae's high lipid content, high growth rate and non-competitiveness with food crops, they were identified as the only possible fuel source to one day replace the entire United States' fossil diesel demands (Chisti 2007). When used in conjunction with CO<sub>2</sub> mitigation, fuel from microalgae can become virtually carbon neutral. Additionally, such microalgal bio-fuel can emit over 70 % less greenhouse gases (SARDI 2009) and it is non-toxic. Nutrient depletion in wastewaters is important to avoid an artificial increase in algal blooms in water bodies.

In order to evaluate if a number of effluent producers are able to reduce nutrients, mitigate carbon dioxide and produce lipids in decentralised algae productions facilities, *Chlorella vulgaris* microalgae were grown in nursery wastewater, municipal wastewater and dairy wastewater in a small scale bio-reactor at the University of Southern Queensland.

The study has proven that *Chlorella vulgaris* microalgae can remove nutrients in nursery, municipal and dairy wastewater. Phosphorous removal rates are most important to avoid artificially triggered algal blooms. Nursery and municipal wastewater showed promising phosphorous removal rates of 0.27 mg/L/d and 0.51 mg/L/d respectively. In particular the municipal phosphorous removal rates showed very promising results with a N:P utilisation ratio of 3:1.

The most successful CO<sub>2</sub> mitigation was found in the nursery wastewater. The algae were able to be fed with CO<sub>2</sub> eight times for 15 seconds per day, where the other two wastewaters could only be fed for 5 seconds per feeding. Therefore, nursery wastewater was believed to be suited as a medium in a CO<sub>2</sub> mitigation operation.

Lipid production was also excellent in the nursery wastewater with a maximum total lipid content of 25.5 %. Municipal wastewater had a lower lipid content of 12.8 %, which was still considered to be good. However, further research into the suitability of these lipids for the bio-diesel production is still required. Unfortunately the amount of harvested neutral lipids in the nursery and municipal wastewater were very low. It was found that approximately 108,000 L of nursery wastewater or 258,000 L of municipal wastewater are required to produce 1 L of bio-diesel. It is believed that larger batch experiments, better extraction techniques and optimisation of the bio-reactor can achieve significantly improved lipid amounts. For example, the experimental error should be significantly reduced if instead of 3.5 L batch experiments 100 L are used.

Generally, the dairy wastewater was found to be unsuitable for nutrient removal (in particular phosphorous). However, further research may reveal improved phosphorous utilisation. Dairy wastewater also performed poor in algal growth, CO<sub>2</sub> mitigation and lipid content. The prime reason for its failure was found to be the water's high turbidity, even though it was heavily diluted with distilled water. Sufficient illumination is the most important growth parameter for algae. If insufficient levels of light are able to penetrate through the wastewater, the algae are likely to perform poorly. Craggs et al (1995) found in their research that during low light intensity days, ammonia removal decreased.

This research also found that even through the 5-day BOD and TOC was kept low and the wastewater was filtered with 0.45 µm filter paper, bacterial growth was non-avoidable. It would be important to find out at what stage amount of bacteria within the wastewater would begin to adversely affect the algal growth. This may be part of future research.

Overall nursery wastewater performed best in terms of algal growth, nutrient depletion, CO<sub>2</sub> mitigation and lipid production. It would be valuable to know whether municipal wastewater can have the ability to achieve similar results as the nursery wastewater if bacterial growth can be reduced. On the whole, this research has proven to be very successful and nutrient depletion, carbon mitigation and lipid production using wastewaters and *Chlorella vulgaris* microalgae is worthy of further research.

## 8.2 SUGGESTIONS FOR FUTURE WORK

### 8.2.1 CONTROLS AND ADDITIONAL EXPERIMENTS

As seen in all batch experiments conducted in this research, bacterial activity and their affect on nutrient transfer played a significant role. Therefore it was difficult to determine what amount of nutrients was actually consumed by the algae. In future research a control reactor should be used to verify the nutrient depletion by the bacteria and algae. In this control, no algae would be inoculated. But it would undergo the same conditions as the inoculated batch. As there will always be some bacterial activity in the wastewater even at reduced temperatures (refrigerator or freezer), proceeding experiments with wastewater from the same source would not be suited for this study. Therefore two exactly the same models of bio-reactor would have to run parallel in order to achieve sufficiently correct results.

Another suggestion for future work is additional experiments for each wastewater. Most of the experiments showed some kind of downfall which could have adversely affected the final results. In order to eliminate 'by chance' results, it would be advisable to repeat each experiment at least three times. Due to time constraints this was not possible during this study.

### 8.2.2 IMPROVED CARBON MITIGATION AND pH CONTROL

Originally the reactor was used for pH measurements of activated sludge using titration of acid and base. The titration data for this experiment could not be used due to the pH interference by the CO<sub>2</sub> addition. That meant that the pH behaviour only gave a correct picture while the pH remained between the tolerance levels. Therefore, in future experiments the acid and base addition should be omitted and instead pH should be solely controlled through CO<sub>2</sub> feeding on demand. That would mean that set feeding times would not be needed and CO<sub>2</sub> would simply be added as the pH rises above a given threshold. This way, the amount of CO<sub>2</sub> addition could be used as a titrimetric measure of CO<sub>2</sub> uptake abilities and algal growth. To achieve this, the algal program used with the LabView software would require some substantial adjustments.

### 8.2.3 REDUCTION OF BACTERIAL COMPETITION

As mentioned in Section 8.2.1, bacterial growth causes difficulty in growing algae and determining the nutrient depletion, growth behaviour, DO and pH fluctuations by the algae. A way to overcome this problem could be the elimination of bacteria. That means that the wastewater requires some treatment before it is used in the bio-reactor and inoculated by *Chlorella vulgaris*. There would be some valuable research opportunities for reducing the bacterial load. It could be carried out through disinfecting the wastewater, applying mechanical methods such as shear forces (certain shear forces have the ability to kill microorganisms, pers. comment T. Yusaf, August 2010) or through other means.

### 8.2.4 IMPROVED REACTOR DESIGN

A significant shortcoming of the current reactor design is its poor light penetration for turbid water. This significantly impacts on the algal growth. Therefore it may also favour bacterial growth rather than allowing the algae to develop successfully. One way to overcome this problem could be a revised bio-reactor design. This could include small tubing or flat panels in which the algae can grow. This design could allow more light penetration.

A revised design of the mixing could also significantly improve algal survival rates. This could include a gentler mixing mechanism (possibly paddle mixing as used in flocculation in water treatment plants), a less vigorous mixing before sampling and less pressure in the air and CO<sub>2</sub> supply. The bubbles resulting from the gas supply can significantly rupture algal cells and result in death. Finer and slower moving gas bubbles may have a positive contribution to algal growth.

### 8.2.5 IMPROVED IDENTIFICATION OF ALGAL GROWTH

Spectrophotometry and suspended solids measurements have shown to provide some promising algal mass indicators in nursery wastewater. However, this technique was less useful for dairy and municipal wastewater. In order to have much better certainty of algal growth it may be necessary to calibrate optical density and suspended solids measurements with cell counting. This was not done for this study due to time constraints.

### 8.3 SUMMARY

This chapter concluded the results of this dissertation and suggested some future research topics. It was found that municipal and nursery wastewaters are likely to be suited for decentralised wastewater polishing, CO<sub>2</sub> mitigation and lipid production. Dairy wastewater was less suited to such a scheme due to poor performance in nutrient depletion and lipid production and due to the need for dilution. A range of suggestions for further research were given such as controls and additional experiments, improved carbon mitigation and pH control, reduction of bacterial load, altered bio-reactor designs and improved algal growth identification parameters.

## REFERENCES

- APHA 1995, *Standard methods for the examination of water and wastewater*, 19th Edition, American Public Health Association, Washington, DC.
- Bassham, JA 2010, Photosynthesis, *encyclopaedia britannica*. Retrieved October 07, 2010, from <http://www.britannica.com/EBchecked/topic/458172/photosynthesis>
- Belcher, H, Swale, E 1978, *A beginner's guide to freshwater algae*, Institute of Terrestrial Ecology, London.
- Benemann, JR, Oswald, WJ 1994, *Systems and economic analysis of microalgae ponds for conversion of CO<sub>2</sub> to Biomass*, Dept. Of Civil Engineering, University of California Berkely. Retrieved December 21, 2009, from <http://www.tpub.com/content/altfuels05/3814/38140273.htm>
- Bott, K, Nayar, S 2008, Bio-diesel from microalgae, *Issues (South Melbourne)*, vol. 85, pp. 39-41.
- Brennan, L & Owende, P 2010, Bio-fuels from microalgae-a review of technologies for production, processing, and extractions of bio-fuels and co-products, *Renewable and Sustainable Energy Reviews*, vol. 14, pp. 557-577.
- Chen, W, Zhang, C, Song, L, Sommerfeld, M, Hu, Q 2009, A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae, *Journal of Microbiological methods*, Vol. 77, Issue 1, pp. 41-47.
- Chisti, Y 2007, Bio-diesel from microalgae, *Biotechnology Advances*, vol. 25, no. 3, pp. 294-306.
- Converti, A, Casazza, AA, Ortiz, EY, Perego, P, Del Borghi, M, 2009, Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for bio-diesel production, *Chemical Engineering and Processing*, vol. 48, Issue 6, pp. 1146-1151
- Craggs, RJ, Smith, VJ, McAuley, PJ 1995, Wastewater nutrient removal by marine microalgae cultured under ambient conditions in mini-ponds, *Wat. Sci. Tech*, vol. 31, no. 12, pp. 151-160.
- Davis, EA 1953, *Laboratory experiments on Chlorella culture at the Carnegie Institution of Washington Department of plant biology, algal culture from laboratory to pilot plant*, p. 113, Carnegie Institution of Washington Publication 600, Washington, D.C.

- De-Bashan, LE, Bashan, Y, Moreno, M, Lebsky, VK, Bustillos, JJ 2002, Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae *Chlorella* spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium *Azospirillum brasilense*, *Canadian Journal of Micro.*, vol. 48, pp. 514-521.
- Ehimen, EA, Sun, ZF, Carrington, CG 2010, Variables affecting the in situ transesterification of microalgae lipids, *Fuel*, vol. 89, pp. 677-684.
- Fogg, GE, Collyer, DM 1953, *The accumulation of lipids by algae, algal culture from laboratory to pilot plant*, p. 177, Carnegie Institution of Washington Publication 600, Washington, D.C.
- Folch, J, Lees, M, Sloane Stanley, GH 1957, A simple method for the isolation and purification of total lipides from animal tissues, *J Biol Chem.*, vol. 226, pp. 497-509.
- Forbes, J & Watson, R 1992, 'Plants as the basis of agriculture', *Plants in agriculture*, Cambridge University Press, Cambridge, pp. 12-31.
- Gonzalez, C, Marciniak, J, Villaverde, S, Garcia-Encina, PA, Munoz, R 2008, Microalgae-based processes for the biodegradation of pretreated piggy wastewater, *Applied Microbiological Biotechnology*, vol. 80, no. 5, pp. 891-898.
- Graham, LE, Wilcox, LW 2000, *Algae*, Prentice Hall, New Jersey, USA.
- Gregory, MJ 2006, *Photosynthesis in Elodea*. Retrieved 31 July, 2010, from <http://faculty.clintoncc.suny.edu/faculty/michael.gregory/files/bio%20101/bio%20101%20laboratory/photosynthesis/photosynthesis.htm>
- Grobbelaar 2004, Algal nutrition, mineral nutrition, *handbook of microalgal culture: biotechnology and applied phycology*, Carlton, Australia.
- Gummert, F, Meffert, ME, Stratmann, H 1953, *Nonsterile large-scale culture of Chlorella in greenhouse and open air, algal culture from laboratory to pilot plant*, p. 166, Carnegie Institution of Washington Publication 600, Washington, D.C.
- Harris, P 2010, *Growth and oil characterisation of Chlorella vulgaris under increased lipid cultivation regimes*, Honours project, Department of Biological and Physical Sciences, Faculty of Sciences, University of Southern Queensland
- Johnson, DA, Sprague, S 1987, *Liquid fuels from microalgae*, Technical Report National Renewable Energy Laboratory (NREL). Retrieved 2 February 2010, from <http://www.nrel.gov/docs/legosti/old/3202.pdf>

- Kebede-Westhead, E, Pizarro, C, Mulbry, WW 2006, Treatment of swine manure effluent using freshwater algae: Production, nutrient recovery, and elemental composition of algal biomass at four effluent loading rates, *Journal of Applied Phycology*, vol 18, no. 1, pp. 41-26.
- Krauss, RW 1953, *Inorganic nutrition of algae, algal culture from laboratory to pilot plant*, p. 85, Carnegie Institution of Washington Publication 600, Washington, D.C.
- Lee, YN, Shen, H 2004, Basic culturing techniques, *handbook of microalgal culture: biotechnology and applied phycology*, Carlton, Australia.
- Li, J 2009, Bio-fuels and water treatment by growing algae in wastewater, *Journal of Australian Water Association*, vol. 36, pp. 92-97.
- Li, Y, Horsman, M, Wu, N, Lan, CQ and Dubois-Calero, N 2008, Articles: biocatalysts and bioreactor design, bio-fuels from microalgae, *Biotechnology Progress*, vol. 24, no. 4, pp. 815-820.
- Lundquist, TJ, 2006, *Production of algae in conjunction with wastewater treatment*, California Polytechnic State University. Retrieved 5 January, 2010 from <http://www.docstoc.com/docs/2988054/Production-of-Algae-in-Conjunction-with-Wastewater-Treatment-Tryg-J>
- Masojidek, J, Koblizek, M, Torzillo, G 2004, *Photosynthesis in microalgae, handbook of microalgal culture: biotechnology and applied phycology*, Blackwell Publishing, Calton, Australia.
- Mata, TM, Martins, AA, Caetano, NS 2010, Microalgae for bio-diesel production and other applications: a review, *Renewable and Sustainable Energy Reviews*, vol. 14, pp. 217-232.
- Miyamoto, K 1997, *Renewable biological systems for alternative sustainable energy production*, FAO Agricultural Service Bulletin 128. Retrieved 17 April, 2010, from <http://www.fao.org/docrep/w7241e/w7241e00.htm#Contents>
- Moheimani, NR 2005, *The culture of coccolithophorid algae for carbon dioxide bioremediation*. Retrieved 15 May, 2010, from <http://researchrepository.murdoch.edu.au/206/>
- Nichols, HW 1973, *Handbook of Phycological Methods*, Ed. JR Stein, Cambridge University Press, Cambridge.
- Neef, W 2007, 'Mit vollgas an die wand', *Wochenzeitung Freitag*, January 2007.
- Nichols, HW 1973, *Handbook of phycological methods*, Ed Stein, JR, pp.16-17, Cambridge University Press, Cambridge.



Park, KY, Lim, BR, Lee, K 2009, Growth of microalgae in diluted process water of the animal wastewater treatment plant, *Water Science and Technology*, vol. 59, no. 11, pp. 2111-2116.

Phillips, JN, Myers, J 1953, Growth rate of *Chlorella* in flashing light, *Plant Physiology*, vol. 29, no. 2, pp. 151-161.

Pienkos, PT 2007, *The potentials for bio-fuels from algae*, Algae Biomass Summit, San Francisco. Retrieved 15 February, 2010, from <http://www.nrel.gov/docs/fy08osti/42414.pdf>

Rodolfi, L, Zittelli, GC, Bassi, N, Padovani, G, Biondi, N, Bonini, G, Tredici, MR 2009, Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor, *Biotechnology and Bioengineering*, Vol. 102, Issue, 1, pp. 100-112.

Poser, HB, Sparrow, AH 1964, Survival of *Chlorella* and *Chlamydomonas* after acute and chronic gamma radiation, *Radiation Botany*, vol. 4, no. 3, pp. 253-257.

Pratt, R & Fong, J, 1940, *Studies in Chlorella vulgaris II. Further evidence that Chlorella cells from a growth-inhibiting substance*, Botanical Society of America.

Putt, R 2007, Algae as a bio-diesel feedstock: a feasibility assessment draft, MSU Department of Chemical Engineering, Auburn, Alabama. Retrieved 15 February, 2010, from [http://bioenergy.msu.edu/feedstocks/algae\\_feasibility\\_alabama.pdf](http://bioenergy.msu.edu/feedstocks/algae_feasibility_alabama.pdf)

Richmond, A 2004, *Biological principles of mass cultivation, handbook of microalgal culture: biotechnology and applied phycology*, Blackwell Publishing, Calton, Australia.

Ruiz-Marin, A, Mendoza-Espinosa, LG, Stephenson, T 2009, Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater, *Bioresource Technology*, vol 101, no. 1, pp. 58-64.

SARDI 2009, *Algal production group, SARDI Aquatic Sciences*, South Australian Research and Development Institute. Retrieved December 20, 2009, from [http://www.sardi.sa.gov.au/aquaculture/aquaculture/microalgal\\_production\\_systems](http://www.sardi.sa.gov.au/aquaculture/aquaculture/microalgal_production_systems)

Sheehan, J, Dunahay, T, Benemann, J, Roessler, P, 1998, *A look back at the U.S. Department of Energy's aquatic species program – bio-diesel from algae*, U.S. Department of Energy's Office of Fuels Development. Retrieved 6 January, 2010, from <http://www.nrel.gov/docs/legosti/fy98/24190.pdf>

- Silvia, A, Figueiredo, SA, Sales, MG, Delerue-Matos, C 2009, Ecotoxicity tests using green algae *Chlorella Vulgaris* – A useful tool in hazardous effluent management, *Journal of Hazardous Materials*, vol. 167, no. 1-3, pp. 179-185
- Sumukhi 2010, *Algae strain selection-a difficult exercise?* Retrieved 20 September, 2010, from <http://www.cleantick.com/users/Sumukhi/blogs/367>
- Travieso, L, Benitez, F, Sanchez, E, Borja, R, Martin, A, Colmenarejo, MF 2006, Batch mixed culture of *Chlorella vulgaris* using settled and diluted piggery waste, *Ecological engineering*, vol. 28, no. 2, pp. 158-165.
- Tsukahara, K, Sawayama, S 2005, Liquid fuel production using microalgae, *Journal of Japan Petroleum Institute*, vol. 48, no. 5, pp. 251-259.
- TWPC (The water planet company) n.d., *Nitrification and denitrification*. Retrieved 2 October, 2010, from [http://www.thewaterplanetcompany.com/docs/WPC\\_Nitrification%20&%20Denitrification%20.pdf](http://www.thewaterplanetcompany.com/docs/WPC_Nitrification%20&%20Denitrification%20.pdf)
- Weber, H 1997, *Woerterbuch der Mikrobiologie*, Gustav Fischer, Ulm, Germany.
- Widjaja, A, Chien, CC, Ju YH 2009, Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*, *Journal of the Taiwan Institute of Chemical Engineering*, vol. 40, no. 1, pp. 13-20.
- Wellinger, A 2009, *Algal biomass, does it save the world?* Retrieved August 29, 2010 from <http://www.iea-biogas.net/Dokumente/AlgaeBiomass8-09.pdf>
- Widjaja, A, Chien, CC, Ju YH 2009, Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*, *Journal of the Taiwan Institute of Chemical Engineering*, vol. 40, no. 1, pp. 13-20.
- Wiggers, VR, Meier, HF, Wisniewski Jr A, Chivanga Barros, AA, Wolf Maciel, MR 2009, Bio-fuels from continuous fast pyrolysis of soybean oil: A pilot plant study, *Bioresource Technology*, vol. 100, no. 24, pp. 6570-6577.
- Woertz, I, Feffer, A, Lundquist, T, Nelson, Y 2009, Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for bio-fuel feedstock, *Journal of Environmental Engineering*, vol. 135, no. 11., pp. 1115-1122.
- Yun, YS, Lee, SB, Park, JM, Lee, CI, Yang, JW 1997, Carbon dioxide fixation by algae cultivation using wastewater nutrients, *Journal of Chemical Technology and Biotechnology*, vol. 69, pp. 451-455.

University of Southern Queensland  
FACULTY OF ENGINEERING AND SURVEYING

**ENG 4111/4112 Research Project**  
**PROJECT SPECIFICATION**

FOR: **Nadine PUFELSKI**  
TOPIC: INVESTIGATION OF *CHLORELLA VULGARIS* ALGAE GROWTH IN DIFFERENT WASTEWATERS FOR SIMULTANEOUS NUTRIENT REMOVAL AND FATTY ACID PRODUCTION

SUPERVISORS: Dr. Vasantha Aravinthan  
ENROLMENT: ENG 4111-S1, 2010;  
ENG 4112-S2, 2010

PROJECT AIM: This project seeks to investigate the suitability of piggery-, iceberg-lettuce processing facility-, and domestic wastewater effluent to act as a growing medium for *Chlorella vulgaris* microalgae. It will explore the algae's capability to remove nutrients and carbon from these different wastewaters, while simultaneously generating lipids for the bio-fuel production.

SPONSORSHIP: TBA

PROGRAMME: **Issue A, 1<sup>st</sup> March 2010**

1. Conduct literature review on the growth of *Chlorella vulgaris* microalgae, its ability to remove nutrients and uptake carbon dioxide from the wastewaters as well as its common fatty acid production yields using different culture media.
2. Collect information on the characteristics of the wastewaters.
3. Develop a suitable growing environment for *Chlorella vulgaris* in different wastewaters and optimise this environment, whilst keeping it closely to natural conditions.
4. Conduct batch experiments to analyse algae growth patterns, nutrient removal and carbon dioxide uptake in the optimised mediums.
5. Analyse the lipid content and fatty acids in the harvested algae.
6. Write-up the final dissertation

*As time permits:*

7. Analyse the suitability of the collected lipids for bio-diesel production.
8. Provide guidelines for future studies in the fields of nutrient removal and lipid production through microalgae

AGREED:

\_\_\_\_\_(Student)\_\_\_\_\_, \_\_\_\_\_(Supervisors)  
\_\_\_\_\_/\_\_\_\_\_/\_\_\_\_\_, \_\_\_\_\_/\_\_\_\_\_/\_\_\_\_\_

Examiner/Co-examiner: \_\_\_\_\_

Nursery 1

Time			Nutrients						Spectrophotometry					Suspended solids			
Date	Time	Time elaps. (d, hrs, min)	TN (mg/L)	NO2-N (mg/L)	NO3-N (mg/L)	Org-N + NH4-N (mg/L)	Nitrification	Change in Org-N + NH4-N	Av. Reading	reactor volume L	Reading x L	concentration change g/L	real reading	Paper Weight g	After Oven g	SS g/20ml	real SS g/L
4/05/2010	17:30	0:00:00	90.640	9.105	24.132	57.403	0.000	16.442	0.09	3.80	0.342	0.090	0.090				
5/05/2010	11:00	0:17:30	68.978	2.551	25.466	40.961	1.282	1.903	0.098	3.78	0.371	0.090	0.098	0.0936	0.0956	0.002	0.098
6/05/2010	11:00	1:17:30	68.357	2.668	26.630	39.059	0.689	1.811	0.121	3.73	0.450	0.092	0.118				
7/05/2010	10:30	2:17:00	67.236	2.698	27.290	37.248	1.839	3.651	0.079	3.66	0.289	0.093	0.076				
10/05/2010	9:00	5:15:30	65.424	2.710	29.117	33.597	-0.368	1.576	0.168	3.60	0.603	0.095	0.159	0.0933	0.0954	0.0021	0.099
11/05/2010	10:30	6:17:00	63.480	2.64275	28.816	32.021	-0.740	2.766	0.189	3.53	0.666	0.097	0.175	0.0931	0.0949	0.0018	0.084
12/05/2010	11:30	7:18:00	59.974	2.133	28.585	29.255	0.286	1.729	0.175	3.46	0.606	0.099	0.159	0.0935	0.0968	0.0033	0.150
13/05/2010	10:30	8:17:00	58.531	2.299	28.705	27.526	0.262	2.035	0.179	3.39	0.609	0.101	0.160	0.0938	0.0961	0.0023	0.103
14/05/2010	12:00	9:18:30	56.758	2.129	29.138	25.491	0.602	-0.808	0.212	3.33	0.705	0.103	0.185				
17/05/2010	9:00	12:15:30	58.168	2.013	29.856	26.299	-31.869		0.104	3.26	0.338	0.105	0.089				

Nursery 2

Time			Nutrients						Spectrophotometry					Suspended solids			
Date	Time	Time elaps. (d, hrs, min)	TN (mg/L)	NO2-N (mg/L)	NO3-N (mg/L)	Org-N + NH4-N (mg/L)	Nitrification	Change in Org-N + NH4-N	Av. Reading	reactor volume L	Reading x L	concentration change g/L	real reading	Paper Weight g	After Oven g	SS g/20ml	real SS g/L
20/05/2010	16:00	0:00:00	110.350	0.521	37.277	72.553	0.000	2.184	0.072	3.64	0.260	0.072	0.072	0.094	0.0956	0.0016	0.080
21/05/2010	9:00	0:17:00	107.945	0.509	37.067	70.369	5.104	5.446	0.059	3.76	0.220	0.069	0.061				
24/05/2010	10:30	3:18:30	107.604	4.342	38.338	64.923	1.030	2.277	0.126	3.63	0.458	0.072	0.126	0.0937	0.0959	0.0022	0.110
25/05/2010	10:30	4:18:30	106.356	5.145	38.565	62.646	0.109	4.005	0.140	3.68	0.513	0.071	0.141	0.0951	0.0972	0.0021	0.106
26/05/2010	13:30	5:21:30	102.460	5.479	38.341	58.640	0.614	2.491	0.227	3.66	0.829	0.071	0.228	0.0952	0.0975	0.0023	0.116
27/05/2010	10:00	6:18:00	100.582	5.379	39.054	56.149	-0.814	4.709	0.285	3.6	1.024	0.072	0.281	0.0944	0.0973	0.0029	0.143
28/05/2010	11:30	7:19:30	95.059	5.159	38.461	51.440	-2.139	6.379	0.426	3.51	1.496	0.074	0.411				
31/05/2010	9:00	10:17:00	86.542	3.880	37.600	45.061	1.898	4.806	0.791	3.28	2.593	0.079	0.712	0.0938	0.0992	0.0054	0.243
1/06/2010	8:30	11:16:30	83.633	7.474	35.904	40.255	-1.669	1.104	0.944	3.13	2.955	0.083	0.812	0.0932	0.0986	0.0054	0.232
2/06/2010	8:00	12:16:00	80.860	7.510	34.199	39.150	-8.380	-6.249	0.941	3.02	2.841	0.086	0.780	0.094	0.1005	0.0065	0.270
3/06/2010	8:30	13:16:30	78.729	0.613	32.716	45.399	-0.315	0.339	0.886	2.86	2.534	0.091	0.696	0.0938	0.101	0.0072	0.283
4/06/2010	8:30	14:16:30	78.075	1.145	31.870	45.061	2.818	5.335	0.875	2.73	2.388	0.095	0.656				
7/06/2010	9:00	17:17:00	75.558	7.028	28.804	39.726	-35.833	39.726	0.320	2.48	0.794	0.105	0.218				

Municipal 1

Time			Nutrients						Spectrophotometry					Suspended solids			
Date	Time	Time elaps. (d, hrs, min)	TN (mg/L)	NO2-N (mg/L)	NO3-N (mg/L)	Org-N + NH4-N (mg/L)	Nitrification	Change in Org-N + NH4-N	Av. Reading	reactor volume L	Reading x L	concentration change g/L	real reading	Paper Weight g	After Oven g	SS g/20ml	real SS g/L
28/07/2010	15:30	0:00:00	27.755	0.250	1.368	26.137	0.000	5.238	0.083	3.86	0.319	0.083	0.083	0.0941	0.0949	0.0008	0.040
29/07/2010	14:00	0:22:30	26.491	4.564	1.027	20.899	0.620	5.465	0.087	4.86	0.425	0.066	0.110	0.0951	0.096	0.0009	0.057
30/07/2010	9:00	1:17:30	21.646	4.906	1.305	15.435	-0.154	4.967	0.069	4.78	0.331	0.067	0.086				
2/08/2010	10:30	4:19:00	16.525	4.774	1.284	10.467	0.233	1.945	0.075	4.15	0.312	0.077	0.081	0.0949	0.0965	0.0016	0.086
3/08/2010	10:00	5:18:30	14.813	4.972	1.318	8.523	-0.511	0.862	0.087	3.99	0.346	0.080	0.090	0.0953	0.0971	0.0018	0.093
4/08/2010	8:00	6:16:30	13.439	4.636	1.142	7.661	-0.043	2.050	0.086	3.82	0.327	0.084	0.085	0.095	0.097	0.002	0.099
5/08/2010	8:30	7:17:00	11.347	4.577	1.159	5.611			0.102	3.65	0.372	0.088	0.096	0.0941	0.0961	0.002	0.095
6/08/2010	9:00	8:17:30							0.101	3.44	0.347	0.093	0.090	0.095	0.0968	0.0018	0.080
9/08/2010	9:30	11:18:00							0.158	2.92	0.461	0.109	0.120	0.0962	0.0992	0.003	0.113

Municipal 2

Time			Nutrients						Spectrophotometry					Suspended solids			
Date	Time	Time elaps. (d, hrs, min)	TN (mg/L)	NO2-N (mg/L)	NO3-N (mg/L)	Org-N + NH4-N (mg/L)	Nitrification	Change in Org-N + NH4-N	Av. Reading	reactor volume L	Reading x L	concentration change g/L	real reading	Paper Weight g	After Oven g	SS g/20ml	real SS g/L
31/08/2010	16:30	0:00:00	116.600	0.139	59.912	56.550	0.000	20.415	0.048	3.84	0.183	0.048	0.048	0.0949	0.0957	0.0008	0.040
1/09/2010	15:00	0:22:30	106.214	0.131	69.949	36.134	-12.221	-18.499	0.082	3.78	0.308	0.049	0.080	0.0942	0.0951	0.0009	0.044
2/09/2010	8:00	1:15:30	112.492	0.128	57.731	54.633	6.737	13.393	0.095	3.63	0.344	0.051	0.089	0.0942	0.0952	0.001	0.047
3/09/2010	10:00	2:17:30	105.836	0.159	64.437	41.240	4.421	5.254	0.133	3.45	0.460	0.053	0.120	0.0956	0.0965	0.0009	0.040
6/09/2010	10:00	5:17:30	105.003	0.454	68.562	35.986	-1.981	2.069	0.130	3.05	0.395	0.060	0.103	0.0945	0.0968	0.0023	0.091
7/09/2010	10:00	6:17:30	100.953	0.685	66.351	33.917	-1.519	-5.788	0.125	2.84	0.355	0.065	0.092	0.0939	0.0952	0.0013	0.048
8/09/2010	15:30	7:23:00	105.222	1.087	64.430	39.705	0.563	2.532	0.134	2.66	0.356	0.069	0.093	0.0943	0.0966	0.0023	0.080
9/09/2010	10:30	8:18:00	103.254	1.433	64.647	37.173	-5.791	2.569	0.138	2.54	0.350	0.072	0.091	0.0967	0.0986	0.0019	0.063
10/09/2010	8:00	9:15:30	94.894	1.917	58.373	34.604	-1.291	2.901	0.170	2.41	0.410	0.076	0.107	0.0933	0.0962	0.0029	0.091
13/09/2010	8:00	12:15:30	90.701	2.995	56.003	31.703	-60.509	33.165	0.196	1.96	0.384	0.094	0.100	0.0943	0.0968	0.0025	0.064

Dairy 1

Time			Nutrients						Spectrophotometry					Suspended solids			
Date	Time	Time elaps. (d, hrs, min)	TN (mg/L)	NO2-N (mg/L)	NO3-N (mg/L)	Org-N + NH4-N (mg/L)	Nitrification	Change in Org-N + NH4-N	Av. Reading	reactor volume L	Reading x L	concentration change g/L	real reading	Paper Weight g	After Oven g	SS g/20ml	real SS g/L
16/08/2010	14:30	0:00:00							0.104	3.79	0.393	0.104	0.104	0.0937	0.0948	0.0011	0.055
17/08/2010	10:00	0:19:30							0.125	3.61	0.452	0.109	0.119	0.0935	0.0945	0.001	0.048
18/08/2010	15:00	2:00:30							0.198	3.38	0.669	0.116	0.176	0.0937	0.0954	0.0017	0.076
19/08/2010	9:00	2:18:30							0.231	3.28	0.757	0.120	0.200	0.0938	0.0954	0.0016	0.069
20/08/2010	10:00	3:19:30							0.236	3.17	0.748	0.124	0.197	0.0935	0.0954	0.0019	0.079
23/08/2010	10:00	6:19:30							0.286	2.71	0.776	0.145	0.205	0.0935	0.0953	0.0018	0.064
24/08/2010	10:00	7:19:30							0.271	2.65	0.717	0.148	0.189	0.0942	0.0959	0.0017	0.059
25/08/2010	15:00	9:00:30							0.279	2.44	0.680	0.161	0.180	0.0942	0.0968	0.0026	0.084
26/08/2010	9:00	9:18:30							0.318	2.33	0.741	0.169	0.195	0.0945	0.0967	0.0022	0.068
27/08/2010	10:00	10:19:30							0.329	2.14	0.704	0.184	0.186	0.0945	0.0974	0.0029	0.082

Dairy 2

Time			Nutrients						Spectrophotometry					Suspended solids			
Date	Time	Time elaps. (d, hrs, min)	TN (mg/L)	NO2-N (mg/L)	NO3-N (mg/L)	Org-N + NH4-N (mg/L)	Nitrification	Change in Org-N + NH4-N	Av. Reading	reactor volume L	Reading x L	concentration change g/L	real reading	Paper Weight g	After Oven g	SS g/20ml	real SS g/L
13/09/2010	10:00	0:00:00	73.015	0.116	0.158	72.742	0.000	4.272	0.123	3.79	0.464	0.123	0.123	0.0947	0.0952	0.0005	0.025
14/09/2010	8:30	0:22:30	68.778	0.134	0.175	68.469	0.077	-2.263	0.132	3.63	0.480	0.128	0.127	0.0947	0.0953	0.0006	0.029
15/09/2010	12:00	2:02:00	71.118	0.196	0.190	70.732	0.147	3.732	0.165	3.58	0.590	0.130	0.156	0.0938	0.0943	0.0005	0.024
16/09/2010	12:00	3:02:00	67.534	0.336	0.197	67.000	0.566	4.872	0.159	3.4	0.539	0.137	0.142	0.0938	0.0943	0.0005	0.022
17/09/2010	14:30	4:04:30	63.228	0.909	0.191	62.127	3.527	7.421	0.177	3.22	0.570	0.144	0.150	0.094	0.095	0.001	0.042
20/09/2010	11:00	7:01:00	59.334	3.951	0.677	54.706	1.618	5.969	0.200	2.78	0.556	0.167	0.147	0.0935	0.0944	0.0009	0.033
21/09/2010	12:30	8:02:30	54.983	3.243	3.003	48.737	2.470	3.449	0.244	2.66	0.649	0.175	0.171	0.0935	0.0945	0.001	0.035
22/09/2010	9:00	8:23:00	54.004	1.560	7.156	45.288	6.101	7.880	0.228	2.57	0.587	0.181	0.155	0.093	0.0942	0.0012	0.041
23/09/2010	11:00	10:01:00	52.225	0.896	13.921	37.409	5.925	5.592	0.286	2.44	0.699	0.190	0.184	0.0937	0.0954	0.0017	0.055
24/09/2010	8:00	10:22:00	52.558	1.521	19.221	31.816	11.186	14.062	0.298	2.32	0.691	0.200	0.182	0.0935	0.0956	0.0021	0.064
27/09/2010	10:30	14:00:30	49.682	2.591	29.338	17.754	6.384	11.196	0.348	1.94	0.675	0.239	0.178	0.0927	0.0954	0.0027	0.069
28/09/2010	10:30	15:00:30	44.871	3.080	35.233	6.559	-6.884	-8.141	0.432	1.82	0.787	0.255	0.208	0.0936	0.0961	0.0025	0.060
29/09/2010	16:30	16:06:30	46.128	2.556	28.872	14.700	-26.452	19.422	0.401	1.64	0.658	0.283	0.174	0.0935	0.0965	0.003	0.065

```
% Matlab Code for DO, pH, Temp in different growth phases
% Author: Nadine Pufelski
% Date: 2010

% general
time_elapsed = xlsread('Acid_and_Base_3_1997.xls', 1, 'Z9:Z28889');
DO = xlsread('Acid_and_Base_3_1997.xls', 1, 'B9:B28889');
pH = xlsread('Acid_and_Base_3_1997.xls', 1, 'C9:C28889');
Temp = xlsread('Acid_and_Base_3_1997.xls', 1, 'D9:D28889');
Dark_Light = xlsread('Acid_and_Base_3_1997.xls', 1, 'AA9:AA28889');
% Acid = ddereq(channel,'r9c8:r28889c8');
% Base = ddereq(channel,'r9c12:r28889c12');

% lag
DO_lag = xlsread('Acid_and_Base_3_1997.xls', 1, 'B2175:B3804');
pH_lag = xlsread('Acid_and_Base_3_1997.xls', 1, 'C2175:C3804');
time_lag = xlsread('Acid_and_Base_3_1997.xls', 1, 'AE2175:AE3804');

% % exponential
% DO_expo = xlsread('Acid_and_Base_3_1997.xls', 1, 'B5435:B7060');
% pH_expo = xlsread('Acid_and_Base_3_1997.xls', 1, 'C5435:C7060');
% time_expo = xlsread('Acid_and_Base_3_1997.xls', 1, 'AE5435:AE7060');

% linear
DO_linear = xlsread('Acid_and_Base_3_1997.xls', 1, 'B11947:B13576');
pH_linear = xlsread('Acid_and_Base_3_1997.xls', 1, 'C11947:C13576');
time_linear = xlsread('Acid_and_Base_3_1997.xls', 1, 'AE11947:AE13576');

% stationary
DO_stat = xlsread('Acid_and_Base_3_1997.xls', 1, 'B18469:B20098');
pH_stat = xlsread('Acid_and_Base_3_1997.xls', 1, 'C18469:C20098');
time_stat = xlsread('Acid_and_Base_3_1997.xls', 1, 'AE18469:AE20098');

% death
% DO_death = xlsread('Acid_and_Base_3_1997.xls', 1, 'B24992:B26621');
% pH_death = xlsread('Acid_and_Base_3_1997.xls', 1, 'C24992:C26621');
% time_death = xlsread('Acid_and_Base_3_1997.xls', 1, 'AE24992:AE26621');
DO_death = xlsread('Acid_and_Base_3_1997.xls', 1, 'B26622:B28251');
pH_death = xlsread('Acid_and_Base_3_1997.xls', 1, 'C26622:C28251');
time_death = xlsread('Acid_and_Base_3_1997.xls', 1, 'AE26622:AE28251');

% general
figure;
plot(time_elapsed, DO)
```

```
grid on
hold on
plot(time_elapsed, Dark_Light)
```

```
figure;
plot(time_elapsed, pH)
grid on
hold on
plot(time_elapsed, Dark_Light)
```

```
figure;
plot(time_elapsed, Temp)
grid on
```

```
% lag
figure;
plot(time_lag, DO_lag)
grid on
```

```
figure;
plot(time_lag, pH_lag)
grid on
```

```
% % exponential
% figure;
% plot(time_expo, DO_expo)
% grid on
%
% figure;
% plot(time_expo, pH_expo)
% grid on
```

```
% linear
figure;
plot(time_linear, DO_linear)
grid on
```

```
figure;
plot(time_linear, pH_linear)
grid on
```

```
% stationary
figure;
plot(time_stat, DO_stat)
```



```
grid on

figure;
plot(time_stat, pH_stat)
grid on

% death
figure;
plot(time_death, DO_death)
grid on

figure;
plot(time_death, pH_death)
grid on

% all
figure;
plot(time_lag, DO_lag, 'r')
grid on
hold on
plot(time_linear, DO_linear, 'b')
hold on
plot(time_stat, DO_stat, 'g')
hold on
plot(time_death, DO_death, 'k')

figure;
plot(time_lag, pH_lag, 'r')
% grid on
hold on
plot(time_linear, pH_linear, 'b')
hold on
plot(time_stat, pH_stat, 'g')
hold on
plot(time_death, pH_death, 'k')

% general
figure (1)
title ('Dissolved oxygen production by Chlorella vulgaris with time')
xlabel('Time [d]')
ylabel('Dissolved oxygen adjusted for 20 degrees Celsius [mg/L]')

figure (2)
title ('pH variation with time')
```

```
xlabel('Time [d]')
ylabel('pH')
axis([0 18 7 7.9])

figure (3)
title ('Temperature variation with time')
xlabel('Time [d]')
ylabel('Temperature [Degree Celcius]')
axis([0 18 18 27])

%Lag
figure (4)
title ('Dissolved oxygen production by Chlorella vulgaris with time on day
2 (lag phase)')
xlabel('Time [h]')
ylabel('Dissolved oxygen adjusted for 20 degrees Celsius [mg/L]')
axis([0 24 4 6.5])

figure (5)
title ('pH variation with time on day 2 (lag phase)')
xlabel('Time [h]')
ylabel('pH')
axis([0 24 7.15 7.55])

% % exponential
% figure (6)
% title ('Dissolved oxygen production by Chlorella vulgaris with time on
day 4 (exponential phase)')
% xlabel('Time [h]')
% ylabel('Dissolved oxygen adjusted for 20 degrees Celsius [mg/L]')
% axis([0 24 3.8 5.2])
%
% figure (7)
% title ('pH variation with time on day 4 (exponential phase)')
% xlabel('Time [h]')
% ylabel('pH')
% axis([0 24 7.2 7.9])

% linear
figure (6)
title ('Dissolved oxygen production by Chlorella vulgaris with time on day
8 (linear phase)')
xlabel('Time [h]')
ylabel('Dissolved oxygen adjusted for 20 degrees Celsius [mg/L]')
axis([0 24 6 12])
```

```
figure (7)
title ('pH variation with time on day 8 (linear phase)')
xlabel('Time [h]')
ylabel('pH')
axis([0 24 7.1 7.6])

% stationary
figure (8)
title ('Dissolved oxygen production by Chlorella vulgaris with time on day
12 (stationary phase)')
xlabel('Time [h]')
ylabel('Dissolved oxygen adjusted for 20 degrees Celsius [mg/L]')
axis([0 24 5 9])

figure (9)
title ('pH variation with time on day 12 (stationary phase)')
xlabel('Time [h]')
ylabel('pH')
axis([0 24 7.2 7.9])

% death
figure (10)
title ('Dissolved oxygen production by Chlorella vulgaris with time on day
17 (death phase)')
xlabel('Time [h]')
ylabel('Dissolved oxygen adjusted for 20 degrees Celsius [mg/L]')
axis([0 24 3.8 5.2])

figure (11)
title ('pH variation with time on day 17 (death phase)')
xlabel('Time [h]')
ylabel('pH')
axis([0 24 7.2 7.9])

% all
figure (12)
title ('Dissolved oxygen production by Chlorella vulgaris with time')
xlabel('Time [h]')
ylabel('Dissolved oxygen adjusted for 20 degrees Celsius [mg/L]')
axis([0 24 3 11])
h = legend('Lag', 'Linear', 'Stationary', 'Death', 2);

figure (13)
title ('pH variation with time')
```

```
xlabel('Time [h]')  
ylabel('pH')  
axis([0 24 7.2 7.9])  
h = legend('Lag', 'Linear', 'Stationary', 'Death', 2);
```